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THE RELATION OF PERIARTERITIS NODOSA TO BRONCHIAL ASTHMA AND OTHER FORMS OF HUMAN HYPERSENSITIVENESS*

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THE first case of periarteritis nodosa was described by Kussmaul and Maier[1] in 1866. This condition was recognized so infrequently that fifty-seven years later (1923) a review of the literature revealed some seventy references to the disease.[2] By 1935, 200 cases were assembled,[3] 1936, 215 cases,[4] 1939, 245 cases,[5] and through 1940, 350 cases.[6] In view of the earlier apparent rarity of reported instances, it is remarkable that some 200 authenticated cases were described from early 1940 through 1943. As a matter of fact, the disease is coming to be recognized so readily that single case reports now occur largely in state journals and other less prominent periodicals, whereas various authors have recently reported series of cases, as many as fourteen in one man's experience.[7]

There have been many theories concerning etiology. That of hypersensitiveness was suggested by Gruber,[8] who in 1925 induced typical arterial lesions experimentally in sensitized animals. Klinge,[9] Masugi and Isabasi,[10] Rich and Gregory,[11] Selye and Pentz,[12] and others likewise succeeded in provoking periarteritis in various species of animals who had been first sensitized and then reinjected. Numerous antigens such as bacteria, horse serum, and tissue extracts were used.

In man, the lesions of periarteritis nodosa have been found at autopsy or on biopsy from patients whose presenting symptoms were those of various forms of allergy. Although this fact had been noted many times, the role of human hypersensitiveness has been treated broadly and no apparent attempt has been recorded to relate periarteritis to the several specific types of hypersensitiveness. These include atopy, atopic-like disorders (intrinsic allergy), drug allergy, serum sickness, bacterial allergy, and contact dermatitis. Although there is dispute as to whether or not some of these are expressions of one underlying process, it matters little to the purpose of this presentation.

Atopy and Atopic-like Disorders (Intrinsic Allergy).—Atopic-like disorders, which include bronchial asthma, chronic urticaria, and chronic vasomotor rhinitis, may be clinically identical to those of atopy. The particular difference is that atopy is mediated by a definite immunologic process with distinctive antibodies and antigens such as foods and pollens that are extrinsic to the body. In nonatopic or atopic-like disorders there is no identifiable immunologic

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mechanism, and the underlying process presumably originates intrinsically. This difference is stressed for the reason that most cases of asthma identified with periarteritis nodosa belong to the latter group.

An analysis by the authors of 300 consecutive cases of periarteritis nodosa, beginning with the first case of bronchial asthma reported some thirty years ago, revealed many instances of associated atopy and atopic-like disorders. Particular attention was paid to bronchial asthma of which there were fifty-four cases, an incidence of 18 per cent. This is a considerably higher incidence than that found by Rackemann and Greene[5] who reviewed the literature up to 1939. Of 229 references, they discovered nineteen cases of asthma to which Rackemann added eight more, giving a total incidence of 12 per cent. It is of interest that during the four years following their report we found twenty-seven more cases, or exactly the same number that had appeared during the period of sixty-eight years embraced by Rackemann and Greene. Furthermore, we found many typical case reports wherein asthma and periarteritis were associated but not confirmed by autopsy or biopsy. Were these included, the incidence of bronchial asthma with periarteritis nodosa would exceed 20 per cent of the reported cases. Many instances of urticaria, vasomotor rhinitis, and other expressions of atopy were encountered. If they were included with asthma, the incidence of atopy and atopic-like disorders accompanying periarteritis nodosa would be well over 25 per cent.

Analysis of case reports reveals that in almost every instance asthma antedated periarteritis; in a few cases, by many years, but in the great majority, by but a few years, and sometimes by only a month or two. In three cases symptoms of asthma followed the onset of periarteritis, but whether or not these were the first attacks of asthma was not stated. In Table I is recorded the ages of patients at the time of onset of periarteritis nodosa. Inasmuch as asthma was then of comparatively short duration in most instances, the age when bronchial asthma first appeared is also roughly recorded in Table I since only decades are stated. It is of interest that the onset occurred before the age of 21 in but 11 per cent, whereas some 70 per cent of all cases of asthma begin in the first two decades.

TABLE I

AGE (IN DECADES) OF PATIENTS WITH PERIARTERITIS NODOSA WITH AND WITHOUT BRONCHIAL ASTHMA

DECADE	ASTHMATIC GROUP		NONASTHMATIC GROUP	
	NUMBER OF CASES*	PER CENT OF CASES	NUMBER OF CASES†	PER CENT OF CASES
First	2	4.3	26	11.6
Second	3	6.4	24	10.7
Third	10	21.3	40	17.7
Fourth	16	34.0	55	24.4
Fifth	6	12.8	38	16.9
Sixth	8	17.0	23	10.2
Seventh	2	4.3	16	7.1
Eighth	0		3	1.3
Average age	35.4 years		35.7 years	

*In seven cases age not stated.
†In twenty-one cases age not stated.

The asthmatic paroxysms were usually severe and rarely were extrinsic allergens as foods and pollens identified as definite etiologic agents. These two facts, coupled with the later age of onset and the hypereosinophilia, identify most of the reported cases as nonatopic or intrinsic asthma.

Perhaps the most remarkable feature of the cases of asthma associated with periarteritis nodosa was the marked hypereosinophilia in the blood (of most). Values exceeding 80 per cent of the total white blood count have been recorded. Of the fifty-four patients with asthma, forty-four had a blood eosinophilia of over 11 per cent, and the majority were far above this value.

TABLE II

TOTAL LEUCOCYTE COUNT AND PERCENTAGE OF EOSINOPHILES IN CASES OF PERIARTERITIS NODOSA AND BRONCHIAL ASTHMA

CASE	AUTHOR	MAXIMUM WHITE BLOOD COUNT	MAXIMUM PERCENTAGE OF EOSINOPHILES
1	Lamb (1914)[13]	20,900	51
2	Laux (1925)[14]	12,600	12
3	Curtis and Coffey (1934)[15]	19,900	37
4	Grill (1934)[16]	20,000	30
5	Taylor and Farley (1934)[17]	25,700	72
6	Middleton and McCarter (1935)[3]	33,950	72
7	Bahrmann (1935)[18]	15,000	60
8	Cohen, Kline, and Young (1936)[19]		30
9	Motley (1936)[4]	32,000	67
10	Leishman (1937)[20]	11,000	55
11	Baehr and Klemperer (1937)[21]	*	*
12	Strong (1938)[22]	32,000	79
13	Fitz, Parks, and Branch (1939)[23]	15,000	50
14	Cabot Case Report (1939)[24]	18,000	43
15	Wier (1939)[25]	45,000	50
16	Rackemann and Greene (1939)[5]	18,000	43
17	Rackemann and Greene (1939)[5]	18,000	47
18	Rackemann and Greene (1939)[5]	26,000	50
19	Rackemann and Greene (1939)[5]	17,000	55
20	Rackemann and Greene (1939)[5]	26,000	70
21	Rackemann and Greene (1939)[5]	50,000	80
22	Rackemann and Greene (1939)[5]	16,000	37
23	Rackemann and Greene (1939)[5]	37,000	70
24	Dawson (1939)[26]		84
25	Trassoff and Scarf (1940)[27]	18,000	11
26	Lebowich and Hunt (1940)[28]	38,200	68
27	Cabot Case 26 (1940)[29]	18,900	47
28	Grant (1940)[6]	21,000	57
29	Coe, Reismann, and DeHoff (1941)[30]	14,600	40
30	Felsen (1941)[31]	32,000	80
31	Tissell (1941)[32]	20,200	49
32	Harkavy (1941)[33]	18,000	40
33	Harkavy (1941)[33]	19,000	39
34	Jones (1942)[7]		77
35	Jones (1942)[7]	14,500	23
36	Lund (1942)[34]		24
37	Baker (1942)[35]	24,550	70
38	Harkavy (1943)[36]	36,000	68
39	Harkavy (1943)[36]	20,000	39
40	Harkavy (1943)[36]	25,000	44
41	Harkavy (1943)[36]	40,000	50
42	Harkavy (1943)[36]	70,000	82
43	Harkavy (1943)[36]	*	*
44	Harkavy (1943)[36]	*	*
	Average	25,860	53.5
	Controls (Cases without asthma)	19,100	2.5

*No blood counts recorded but hypereosinophilia stated to be present.

Of the ten patients with asthma without eosinophilia,[3, 44-46] no differential blood counts were recorded in seven, and increased eosinophiles may well have been present. In each of the remaining three instances only one count was reported with values of 6 per cent, 4 per cent, and 4 per cent of eosinophiles, respectively. Haining and Kimball[37] recommended repeated differential counts lest hypereosinophilia be missed. In a case of periarteritis nodosa and asthma reported by Lebowich and Hunt[28] there was no abnormal rise in eosinophiles until the disease was well advanced, when they appeared in large numbers. Of the 151 patients with periarteritis nodosa without asthma in which differential counts were recorded, but nine instances of hypereosinophilia appeared (Table III). This gives an incidence of 6 per cent in distinction to 94 per cent of the forty-seven cases with associated asthma.

TABLE III

CASES OF PERIARTERITIS NODOSA AND HYPEREOSINOPHILIA WITHOUT ASTHMA

CASE	AUTHOR	TOTAL WHITE BLOOD COUNT	PER CENT OF EOSINOPHILES	REMARKS
1	Wohlwill (1923)[39]	"Normal"	16	Two other blood counts—normal values for eosinophiles; very inadequate history; examination of lungs not recorded
2	Haining and Kimball (1936)[37]	16,500	33	
3	Cabot Case Report (1936)[38]	35,000	35	Cough for years
4	Sandler (1938)[40]	21,000	55	Sniffles and nasal mucoid discharge
5	Wegener (1939)[41]	2,200	20	
6	Neumann (1940)[42]	–	68	Very brief report, examination of lungs not recorded; urticaria
7	Grant (1940)[6]	35,000	55	Nasal polyps and râles in lungs, but nature of these not mentioned
8	Payne (1941)[43]	17,050	17	
9	Jones (1942)[7]	18,000	28	

Hypereosinophilia of over 15 per cent in adults with uncomplicated bronchial asthma is unusual. Consequently, the association of bronchial asthma and a marked hypereosinophilia should lead to a strong suspicion of an underlying periarteritis nodosa.

Drug Allergy.—The production of periarteritis nodosa by drugs is, as yet, supported by little data. Rich and Gregory[47] observed a series of patients with pneumonia who received relatively large amounts of therapeutic serum together with sulfonamide drugs. At autopsy, typical lesions of periarteritis nodosa were found. The participation of the underlying infection was excluded by reproducing similar lesions in rabbits by injections of horse serum and sodium sulfadiazine. Although the experiment was successful with the administration of horse serum alone, Rich and Gregory encountered one patient who manifested typical arterial lesions after he had received sulfonamide without serum. In this case, tissue removed by biopsy at five months and one week before the sulfonamide reaction showed no periarteritis, whereas tissue study revealed typical widespread lesions nine days following the drug reaction. It is difficult to ascribe such a train of events to anything but drug allergy.

The question as to whether periarteritis caused by a sulfonamide drug is due to a hyperergic reaction or to a direct toxic effect will not be discussed

here, since it introduces the general problem of the pathogenesis of such drug reactions. However, the work of Hageman and Blake[48] and others[49] indicate that hypersensitiveness is the underlying mechanism.

Serum Sickness.—In 1937 Eason and Carpenter[50] found the lesions of periarteritis nodosa at autopsy of a patient who had had acute rheumatic fever treated with antistreptococcal serum. These authors believed that the vascular lesions were due probably to the rheumatic process rather than to the serum. In the same year Clark and Kaplan[51] reported two patients who developed serum sickness following the administration of large amounts of serum. They, too, presented periarteritis nodosa at post-mortem examination. Not only were there necrotising arteritis and periarteritis of the smaller coronary arteries, but proliferation of histiocytes in the mural and valvular endocardium. The following year Clark reported another case, one of acute poliomyelitis, in which a single dose of concentrated horse serum had been given both intraspinally and intramuscularly.

It was Rich and Gregory,[11] however, who demonstrated both clinically and experimentally that typical, extensive periarteritis nodosa may be produced by injections of foreign serum. They succeeded in causing the lesion to appear in rabbits following a single large injection of horse serum. In such instances, free antigen remained in the blood stream after specific antibodies had developed.

Rich and Gregory's observations are surprising in that after forty years of serum therapy, they were the first to identify, definitely, periarteritis as a consequence of serum treatment. There doubtless have been thousands of autopsies on patients who had received therapeutic sera and died soon thereafter, and yet the lesions of periarteritis nodosa, which are too obvious to escape detection, were described first in 1937. Rich and Gregory's series of cases is, therefore, remarkable. Two possibilities concerning the production of periarteritis in these cases present themselves. One involves the question as to whether sulfonamide drugs enhanced the action of serum on vascular tissues. Rich and Gregory commented on this possibility but believed it improbable because they were able to produce typical lesions in rabbits with serum alone just as readily as when the drug was used in addition. The second possibility concerns the large amounts of serum that were given both clinically and in experimental animals, a fact which the authors also recognized, and they pointed out that Clark and Kaplan's two reported patients likewise were given serum in large quantities. Although both precipitins and "serum sickness antibody" are usually present in the blood, attempts to correlate immune reactions with the extent of periarteritis present were not made in any of the cases studied.

Bacterial Allergy.—Bacterial allergy is exemplified by such lesions as the tuberculin skin reaction, rheumatic fever, and the "id" of various fungus infections. The term infectious asthma is often used on the assumption that asthma in the presence of respiratory tract infection is, likewise, due to bacterial hypersensitiveness, although proof of this theory is lacking. Certainly the mechanism involved in such cases of asthma is obscure and is different from those due to atopy wherein an antigen-antibody reaction is demonstrable. Of importance insofar as periarteritis nodosa is concerned is the consideration as to whether the lesions of acute rheumatic fever are a hyperergic response

to the product of streptococcal growth or to other antigens. Swift and his associates[52] assembled evidence to support this theory and it has gained wide acceptance.

Rheumatic fever and periarteritis are linked together by three facts. One concerns the frequency with which both clinical symptoms of rheumatic fever and typical Aschoff bodies have been found with periarteritis nodosa. Klinge,[9] Friedberg and Gross,[53] Spiegel,[44] and others have noted this association. The second fact is that in instances of severe rheumatic fever with involvement of the blood vessels, rheumatic arteritis is at times indistinguishable from periarteritis nodosa. In each there may be fibrinoid degeneration, palisading, and necrosis of the intima and signs of intense perivascular inflammation. One possible distinction has been commented upon:[54] namely, that with nonbacterial allergens, eosinophiles are numerous, whereas in rheumatic arteritis they are not, but the necrosing tissue is eosinophilic. This point needs elaboration although Rich found eosinophiles in his experimental animals. Finally, lesions of both rheumatic fever and periarteritis have been produced experimentally by injections of foreign serum into rabbits. Klinge[9] observed arteritis which he believed resembled that of rheumatic fever as well as a vascular lesion simulating periarteritis after repeated injections of horse serum. Metz[55] induced periarteritis nodosa by injection of cattle serum and recognized rheumatic lesions in some of his animals. Rich recently discovered typical Aschoff bodies in rabbits injected with a large amount of horse serum.

These experiments wherein the typical Aschoff bodies of rheumatic fever have been produced through the process of sensitization with nonbacterial antigens have important implications. They strongly support the theory that the lesions of rheumatic fever express a hyperergic reaction and they may reveal the mechanism, as yet unknown, of bacterial hypersensitiveness.

Although, clinically, periarteritis nodosa itself presents signs of infection such as fever, acute arthritis, and usually a marked leucocytosis, no specific infectious agent has been identified with it.

Contact Dermatitis.—We encountered no case of contact dermatitis with periarteritis nodosa. This is not surprising since the eczematous lesion of contact dermatitis is confined largely to the skin epithelium where the only blood vessels present are capillaries.

DISCUSSION

In previous reports dealing with hypersensitiveness and periarteritis nodosa, no consideration has been given to the identification of the particular types of allergy involved. Rich implied certain forms by stating that the arterial lesions resembled "hives of the blood vessels." Such a lesion (whealing) occurs in atopy and atopic-like disorders, serum sickness, and, occasionally, in drug reactions. It is by no means typical of bacterial allergy.

Previous to this investigation no apparent factor common to all forms of human hypersensitiveness had been apparent. It is of interest that periarteritis nodosa appears to be the only evident manifestation which links all types of human hypersensitiveness together with the exception of contact dermatitis. This fact points again to the role of the blood vessels in hypersensitive states,

Roessle[56] demonstrated that the vasculature is profoundly affected in sensitized animals. A drop of horse serum applied to the mesentery of a frog previously injected with that antigen showed pronounced capillary changes. Abell and Schenck[57] demonstrated marked contraction of the arteries of the ears of sensitized rabbits when they were reinjected with homologous antigen. In wheals, the common lesion of many allergic disorders, there is marked dilation and increased permeability of the capillaries.

These observations may be linked to the circumstances under which periarteritis nodosa, a rare complication, follows asthma. One possible clue resides in the fact that in the series of cases in this report, asthmatic paroxysms were usually of marked intensity. One characteristic of all forms of hypersensitiveness with the exception of some bacterial types is their reversibility. After seizures of asthma or hay fever, for instance, at least in the early stages, the tissues involved appear entirely normal after the attacks cease. This is particularly true of urticaria. No matter how extensive whealing may be or how often repeated, there is complete reversibility to normal skin.

It would appear that possibly the intensity of the asthmatic attacks, or rather the intensity of the underlying mechanism that causes them, may lead to irreversible reactions in the arteries. This possibility is suggested by a similar circumstance that occurs in rheumatic fever. It is believed that the vasculature participates generally in each acute attack. The process is reversible except after intense attacks occur repeatedly when permanent rheumatic arteritis may occur. As this lesion progresses it may closely resemble periarteritis nodosa.

This transition from hypersensitive reversible reactions to nonreversible ones is typified by the Arthus phenomenon. A rabbit injected repeatedly with horse serum and tested intradermally with this antigen will at first develop a typical wheal which disappears completely. On subsequent intradermal injection a large wheal appears which subsides more slowly. Further testing induces greater whealing, then cellular induration, and finally the process is no longer reversible and tissue necrosis occurs. At this stage, necrosis of arteriolar wells appears, as pointed out by Gerlach.[58] The similarity of allergic processes that lose their reversibility to the Arthus phenomenon has been noted repeatedly.[11, 59]

The identification of most cases of periarteritis nodosa with nonatopic or intrinsic asthma is interesting in one respect. The underlying mechanism of this type of asthma is as yet unknown. One prevailing theory is that large amounts of histamine (or H-substance) is released by the tissues, and this substance causes the local lesions. The same mechanism presumably operates in atopy when antigen comes in contact with specific antibody, but as a rule the symptoms of atopic asthma are less intense than in the nonatopic type. That histamine released in excessive amounts may likewise be responsible for the lesions of periarteritis nodosa is suggested by the experiments of Bahrmann,[18] who produced typical arterial changes in rabbits by repeated large injections of histamine.

The relationship between periarteritis nodosa and hypersensitiveness has attracted the attention of numerous investigators, especially in the past few years. Most of these studies have been approached from a pathologic view-

point without regard to specific types of hypersensitiveness. This presentation has utilized such material in an attempted correlation with clinical allergy. Much work remains to be done to make this association complete.

SUMMARY

1. In 300 consecutive cases of periarteritis nodosa bronchial asthma was identified in fifty-four, or 18 per cent.

2. When differential blood counts were available, all but three of forty-seven cases of asthma (94 per cent) showed a hypereosinophilia ranging from 11 to 84 per cent, with an average of 53.5 per cent. This is in marked contrast to 151 cases without asthma in which there were but nine instances of hypereosinophilia (6 per cent) and the average eosinophile count was 2.5 per cent.

3. The association of periarteritis nodosa to the various forms of human hypersensitiveness is discussed.

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THE DEMONSTRATION OF ANTI-RH AGGLUTININS—AN ACCURATE AND RAPID SLIDE TEST

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IT IS now generally appreciated that at least two types of medical difficulties result from the development of antibodies against the RH factor in the sera of Rh-negative individuals: intragroup hemolytic transfusion reactions following the use of Rh-positive blood cells and erythroblastosis fetalis or hemolytic anemia of the newborn in the offspring of sensitized women. In both types of cases, the laboratory demonstration of anti-Rh agglutinins is desirable. In tests upon the blood of recipients of transfusions, detection of such agglutinins may anticipate the possibility of a hemolytic response or resolve the otherwise inexplicable occurrence of such a reaction. The identification of anti-Rh agglutinins in the blood sera of women may anticipate or substantiate development of erythroblastosis fetalis in the infant.

Clinical and laboratory experience has shown that present methods of demonstrating anti-Rh agglutinins in the sera of sensitized individuals are inadequate and the results often misleading.

The two methods in common use are both dependent on the assumption that a serum containing these abnormal agglutinins when mixed in a test tube with a 2 per cent suspension of Rh-positive red cells and incubated for an hour at 37° C. will agglutinate such cells. This is the basis for the "modified compatibility test"[1] which has been advised and employed when Rh typing of the recipient and the donor cannot be done or when only Rh-positive blood is available for transfusion of an Rh-negative individual. It is expected that failure of agglutination of donor's cells by recipient's serum after this lengthy incubation in a test tube indicates that the contemplated transfusion is safe. This method, while effectual in some instances, may give a misleading negative result in 50 per cent or more of the cases tested, thus failing to disclose the probability of in vivo agglutination of Rh-positive cells by the recipient's serum and giving the physician a false assurance of safety. This has been proved clinically in a large number of cases studied in the Blood Grouping Laboratory. One such case may serve as an illustration:

Patient 1 was a Group A, Rh-negative woman married to a Group O, Rh-positive, homozygous man. She was the mother of two Rh-positive children. The first was normal, but the second had had severe, typical erythroblastosis fetalis. A third pregnancy had been aborted at three months, and in a fourth pregnancy, a hysterotomy and sterilization were carried out at the end of the third month. The fetal blood proved to be strongly Rh positive. Following the operation, a transfusion was given. The patient's serum contained agglutinins

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of high titer but of specificity anti-Rh′ (that is, agglutinating Rh′ and Rh_1 cells but not Rh_2 cells).* Prolonged incubation with Rh_2 cells in the test tube, the "modified compatibility test," failed to demonstrate any agglutination. It was therefore decided to attempt cautiously a small transfusion and 150 c.c. of Group A, Rh_2 cells were given. The first 30 c.c. were injected very slowly, during the course of thirty minutes. No symptoms of untoward reaction were noted, so the next 120 c.c. were administered in the course of another half hour. About one and one-half hours after the transfusion had been started, the patient developed a chill, pain in the flanks, and subsequent high fever. The urine, which had previously been entirely normal, four hours later contained some red cell casts and a trace of albumin. The patient was given large amounts of alkaline fluids by mouth and seemed entirely recovered at the end of forty-eight hours. Her further convalescence was uneventful.

This patient had been known to have in her serum an anti-Rh agglutinin of specificity anti-Rh′ (agglutinating Rh′ and Rh_1 cells but not Rh_2 cells—sometimes called a 70 per cent serum since it agglutinates the red cells of approximately 70 per cent of the white population). Because it was of such high titer, this serum had been used for many months in the Blood Grouping Laboratory in parallel tests with an Rh_0 agglutinin for differentiation between Rh_1 and Rh_2 cells. It had not agglutinated any Rh_2 cells in the test tube in thousands of tests. When, before transfusion of this patient, prolonged incubation of her serum with Rh_2 cells showed no agglutination, it was deemed safe to use this blood. An hour and a half after 150 c.c. had been administered a typical hemolytic reaction occurred. Fortunately, so little blood had been used that recovery was complete. Here, as in many subsequent cases studied, the incubation test failed to anticipate an in vivo agglutination.

Further evidence of the inadequacy of this method of demonstration of anti-Rh agglutinins is the well-recognized fact that between 30 and 50 per cent of Rh-negative mothers of Rh-positive babies with definite signs of erythroblastosis fetalis show by the incubation test either no or, at most, a very low titer of anti-Rh agglutinins. Some women in this group have given birth to infants showing such degree of damage that agglutinins of high titer might have been expected in the maternal serum. In fact, it has been noted that mothers of infants with universal edema and icterus gravis (the two severe forms of congenital hemolytic anemia of the newborn) most frequently fall into this category of poor (or apparently poor) anti-Rh agglutinin carriers.

The incubation method for demonstration of anti-Rh agglutinins as carried out in this laboratory involves the use of 2 per cent suspensions of eleven different Group 0 cells. Of these, five are Rh_2, five are Rh_1, and one is Rh negative. Five different cells of two specificities are used because, as we have already shown,[3] cells vary in their agglutinability with different sera. For example, we have found many sera to give only trace or 1 plus reactions with most Rh_2 cells and 4 plus reactions with most Rh_1 cells. Other sera give the opposite reaction. The Rh-negative cell, of course, serves as a control. To two drops of these suspensions are added two drops of the serum to be tested. The mixture is then incubated for one hour at 37° C., shaken well, and centrifuged for one minute at 500 r.p.m. Agglutination is then searched for both macroscopically and microscopically. In recent months, we have also employed a modification

*Nomenclature of Wiener.[2]

of the "blocking test" as outlined by Wiener.[4] For this test, 2 per cent suspensions of Rh_1 and Rh_2 cells are employed. Two drops of the unknown serum are added to two drops of the suspensions. One drop of a standard (titer 4 to 8) anti-Rh_0 serum, known to produce 4 plus agglutination of the test cells, is then added to the mixture. Incubation, centrifugation, and search for agglutination are subsequently carried out as in the case of the anti-Rh tests.

The following case report will serve to illustrate the sort of test tube results frequently obtained by these methods in the severer forms of erythroblastosis fetalis:

Patient 2 was a Group A, Rh-negative woman married to a Group 0, Rh-positive, homozygous man. Her first child, born in 1934, was normal. The second pregnancy (1936) resulted in the stillbirth two weeks before term of an infant with intense icterus and moderate edema. The third pregnancy (1937) also resulted in the stillbirth, four weeks before term, of a baby with icterus and severe edema. In 1940, the patient again delivered a stillborn with severe edema four to six weeks before term. And in 1942, in the seventh month of pregnancy, she once more delivered a stillborn with most extensive hydrops fetalis. This woman's serum tested repeatedly from 1940 to 1942 was never shown to contain more than traces of anti-Rh agglutinins by ordinary methods. The incubation test at the present time shows only traces of agglutinins.

This Rh-negative woman, married to an Rh-positive man, had a normal first-born child. Four succeeding pregnancies resulted in infants with severe hemolytic anemia of the newborn with intense icterus and increasing and earlier fetal hydrops. Certainly her serum might have been expected to contain powerful anti-Rh agglutinins, since succeeding pregnancies resulted in greater and earlier damage to each infant. Yet repeated attempts to demonstrate anti-Rh agglutinins in the test tube never showed more than trace reactions and often were unsuccessful. Obviously in this patient and in many others with a similar history, this method of detecting these abnormal agglutinins failed.

Additional cause for dissatisfaction with the present method of testing is found in our previously reported observations[3] upon a serum pool in which, by the incubation method, anti-Rh agglutinins were not demonstrable. In this experiment, a measured amount of this "inactive" serum was given intravenously to an Rh_1rh individual. Tests of the patient's blood after injection showed some of the red cells to be bound by inhibitor substance and therefore unagglutinable in the test tube by any other Rh_0 serum. At the same time the patient's serum showed the presence of free anti-Rh_0 agglutinins. In other words, the serum previously regarded as inactive was separated in the patient's blood stream into an inhibitor substance which attached itself to the red cells and an agglutinin which at this time was free in the serum. In vitro absorption experiments corroborated this in vivo finding: When the subject's initial cells were added to the inactive serum in a ratio of 1 or more parts of serum to 1

TABLE I

EFFECT OF ADDING POOLED "INACTIVE" SERUM TO Rh_1rh CELLS

Part of Serum	10------1	1	1	1	1	1	1	1	1	1
Part of Blood	1------1	2	3	4	5	6	7	8	9	10
		Degree of Agglutination								
	0------0	±	+	++	+++	++++	++++	HEMOLYSIS		

part of blood, there was no agglutination, but when an increasing concentration of red cells was tested, agglutination became more and more apparent. With a ratio of 1 part serum to 6 parts blood, agglutination was 4 plus. Beyond this concentration of red cells, hemolysis occurred. This result is illustrated in Table I.

These observations led to the examination of several anti-Rh′ sera which we had found to be like those described by Race;[5] that is, capable of inhibiting the action of anti-Rh_0 sera on Rh_2 cells but incapable of producing agglutination when added in excess to a 2 per cent suspension of such cells. In this case, at incubator temperatures (37° C.) no demonstrable agglutination occurred,

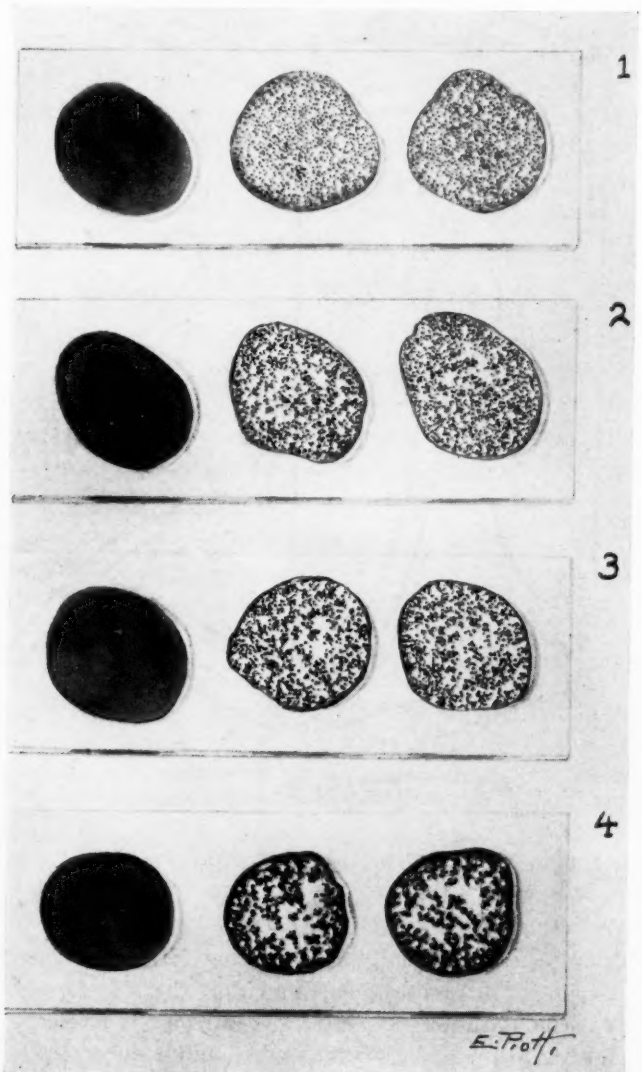

Fig. 1.—Four slides illustrating agglutination of varying strength. In each case, control, unagglutinated, Rh-negative blood is on the left, Rh_2 blood in the center, and Rh_1 blood on the right. No difference between the subtypes of Rh-positive cells is demonstrable in the final agglutination. *1*, 1 plus agglutination; *2*, 2 plus agglutination; *3*, 3 plus agglutination; *4*, 4 plus agglutination.

even after several hours, but at room (20° C.) and at refrigerator (4° C.) temperatures, agglutination and, eventually, hemolysis took place. Subsequent incubation did not break up the agglutinates, thus supporting the assumption that Rh, and not "cold," agglutinins were responsible for the reaction.

The question immediately presented itself as to whether inactive sera from mothers of erythroblastotic infants would not behave in the same way, and upon investigation this was found to be the case. That is, sera from sensitized women in which no anti-Rh agglutinins could be demonstrated by the ordinary

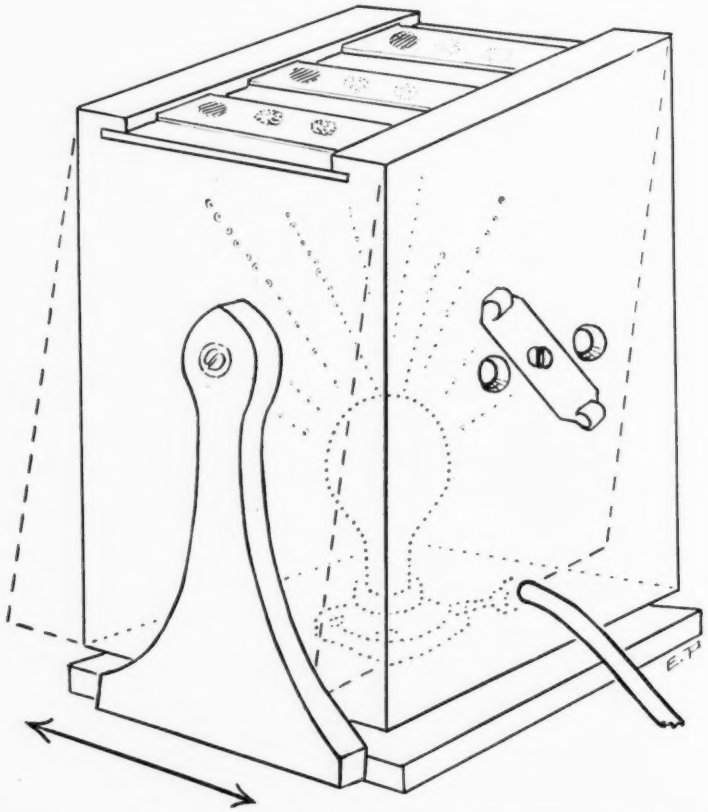

Fig. 2.—Illuminated box with ground-glass top for rocking slides and viewing agglutination. Air vents on both sides dissipate some of the heat from a 40-watt bulb and keep the ground-glass surface at about 37° C.

method, were capable of agglutinating and even of hemolyzing Rh-positive cells when such cells were added in excess. Sera from Patients 1 and 2, previously described, provided two of our test cases.

The conclusion seemed justified, therefore, that certain sera contained agglutinins masked by an inhibitor substance* and that the interference of this substance with the demonstration of agglutination was favored by dilution of agglutinogen (that is, Rh-positive erythrocytes) and incubation.

These observations suggested a simple test, which has now become standard in our laboratory, for detection of anti-Rh agglutinins: Approximately 0.2 c.c.

*The inhibitor substance is fully discussed elsewhere.[3-5]

of fresh, oxalated, Rh-negative, Rh_2 and Rh_1 Group O whole blood or washed cells are placed upon an ordinary slide. Cells of different specificity are used for the same reason as with the test tube method. If washed cells are used, the suspension is made up to approximately 40 to 50 per cent. With each of these is mixed 0.1 c.c. of the serum to be tested. The mixture is gently rotated or repeatedly tilted. The reaction seems to be accelerated when the slide is warmed to 37° C. or thereabouts, but it is easily performed at room temperature. Reading of the test is facilitated by holding the slide on a piece of ground glass placed over an ordinary electric light, but a mirror or white surface also serves to reflect sufficient natural or artificial light through the slide to reveal agglutination from its onset. When the serum is from a sensitized individual, usually within a minute, and certainly within three minutes, plainly visible agglutination appears in one, and usually in both, of the Rh-positive bloods. The Rh-negative blood serves as a control to eliminate errors through mistaking fibrin shreds, rouleaux formation, or nonspecific agglutination for actual Rh agglutination. Rouleaux may be broken up by adding a drop of saline and stirring it into the mixture of blood and serum. Saline does not break up agglutinates but rather makes them stand out more clearly. Fibrin is eliminated in large part by using oxalated test blood, but in the event a drop of finger blood is used for testing, the oxalate may be added to the serum. It should be emphasized that in order to obtain a satisfactory slide test, one must follow carefully the principle that a thick drop of blood is necessary. If thin suspensions are used on the slide, the results will be equivocal. This may be illustrated by the following case: Serum from Patient 2 contained no demonstrable anti-Rh agglutinins in the test tube, but the slide test was positive 4 plus. When used with increasingly thinner cell suspensions, the results shown in Table II were obtained.

TABLE II

EFFECT OF DILUTING RED CELLS ON THE SLIDE TEST

Strength of red cell suspension	50%	40%	30%	20%
Degree of agglutination with anti-Rh serum	++++	+++	++	0

Suspensions heavier than 50 per cent may be used, but the clumps are usually so close together as to be hard to visualize. A drop of saline, of course, will overcome this difficulty. Suspensions of red cells less than 30 per cent may show agglutination, but this will vary with the type of agglutinin and its concentration in the serum. For these reasons 40 or 50 per cent suspensions of red cells have been found most satisfactory.

The test as outlined has been devised to meet the needs of a laboratory evaluating many specimens of blood serum each day. For practical clinical purposes, it is not necessary to measure the cells and serum exactly. Suitable proportions are obtained when a large drop of blood from an ordinary finger prick is mixed with approximately half as much serum. Nor is it necessary to be particularly concerned about specificity of the red cells used if the test is set up with several different specimens of Rh-positive blood. If the mother's blood is of the same group and therefore contains no isohemagglutinins against the infant's or the father's blood, it is suggested that theirs be used as the

test cells. The mother's cells, of course, may conveniently be employed as the Rh-negative control. The reaction with the infant's cells alone, if negative, against the mother's serum should not be depended upon, because it is possible in some cases that a high proportion of the baby's cells may be combined with inhibitor substance and fail to agglutinate. In cases of possible blood incompatibility between donor and recipient, the recipient's serum may be set up on a slide with the donor's fresh oxalated or citrated cells.

Until more information is available concerning the nature of the inhibitor substance and its mode of action, it is not possible to state with certainty the mechanism of the slide test. However, it seems likely that two factors, an agglutinin and an inhibitor substance (both capable of attachment to cells and both therefore presumably antibodies), exist independently in most sera. When inhibitor substance is present in nearly equivalent or stronger titer than agglutinin and a thin cell suspension is used, most of the red cells are bound by inhibitor substance, leaving so few cells to be bound by agglutinin that visible clumps cannot be formed. If, on the other hand, large numbers of red cells are used, enough erythrocytes are present both to absorb the inhibitor substance and to combine with agglutinin in sufficient number to form easily visible clumps.

We have found by the slide test method that all our sera which had been classified here and elsewhere as anti-Rh′ are capable of agglutinating Rh_2 cells on the slide, and sera classified as anti-Rh_0 are capable of agglutinating Rh′Rh′ cells by this method. We have one anti-Rh″ serum which does not agglutinate Rh_1 or Rh′ cells in this way.

Using the slide test, serum from Patient 1 gives strongly positive agglutination with Rh_2 cells. Therefore, although no agglutination of such cells is apparent in the test tube, the patient has strong agglutinins against such cells in her serum and the transfusion reaction resulting from the infusion of Rh_2 cells is readily understandable. Serum from Patient 2 also shows the presence of strong anti-Rh agglutinins on the slide, and the fact that she has given birth to children with severe erythroblastosis fetalis is no longer puzzling.

To date more than 250 slide tests have been performed in parallel with test tube tests for anti-Rh agglutinins and inhibitor substance. Of these, ninety have been on sera from mothers of erythroblastotic babies. By the usual incubation technique, only thirty-seven of these sera were found to contain anti-Rh agglutinins. The slide test was positive in all of these cases. The test for inhibitor substance in sera from sensitized individuals was in most cases positive when the usual test for anti-Rh agglutinins was negative or weak. In a few cases, however, the agglutinin and inhibitor substance present in the mother's serum appear to be so nearly balanced that only by elaborate testing, with careful control of all variables, can either be demonstrated by the test tube method.

In addition to the ninety cases mentioned above, thirty-eight of our stored sera, known to contain anti-Rh agglutinins, have been retested. The slide test was strongly positive, whereas the incubation test showed varying degrees of agglutination.

As an evidence of Rh sensitization, three methods of testing for antibodies induced by the Rh antigen have been used: namely, the slide test, the incubation test, and the test for inhibitor substance interfering with the incubation

agglutination technique. In tests of ninety consecutive cases of probable Rh sensitization the slide test confirmed the presence of antibodies in a higher percentage of cases than either of the other two tests. This is illustrated, for twenty-two of the sera tested, in Table III.

It can readily be seen that in these twenty-two cases, sensitivity was indicated by the incubation test for agglutinins in thirteen, by the "blocking test" in sixteen, and by the slide test in twenty-two. The results of the combined incubation and blocking test indicated sensitization in twenty-one of the twenty-two. It is not unlikely that if appropriate conditions are provided, test tube

TABLE III

COMPARISON OF THE SLIDE TEST WITH OTHER TESTS FOR RH SENSITIZATION

TYPE OF CELLS	SLIDE TEST			TEST TUBE TEST			"BLOCKING TEST"		
	1	2	3	4	5	6	7	8	9*
	Rh_1	Rh_2	rh	Rh_1	Rh_2	rh	Rh_1	Rh_2	Control
Serum 1	+++	+++	0	0	0	0	0	0	++++
2	+++	++++	0	++++	+++	0	++++	++++	++++
3	++++	++++	0	0	0	0	0	++	++++
4	++	++	0	+	++	0	++	++	++++
5	++++	++++	0	++++	++++	0	++++	++++	++++
6	++++	++++	0	++++	++	0	++++	++	++++
7	++	++	0	0	0	0	0	+	++++
8	++	+±	0	++	++	0	++	+	++++
9	++++	++++	0	+	Tr	0	++++	++++	++++
10	+	++	0	+++	+	0	++++	++++	++++
11	++	+++	0	0	0	0	0	0	++++
12	+++	++	0	+++	0	0	+++	++	++++
13	+±	+±	0	0	0	0	++++	++++	++++
14	++	++	0	0	0	0	0	0	++++
15	+++	+++	0	++	0	0	++++	0	++++
16	++++	++++	0	++	++	0	+±	++	++++
17	+	+++	0	++	+	0	++++	++++	++++
18	+++	++++	0	0	0	0	0	0	++++
19	++++	++++	0	0	0	0	0	+++	++++
20	++	+++	0	0	0	0	0	0	++++
21	+++	+++	0	+	+++	0	+	++++	++++
22	++++	++++	0	0	0	0	0	+++	++++

*The "blocking test" described involves the mixture of Rh+ cells with unknown serum, followed by addition of a serum known to produce strong agglutination in the test tube. Therefore, 4 plus reactions in columns 7 and 8 mean no "blocking" or inhibitor substance; 1 plus to 3 plus results mean partial inhibition; and a negative result means that strong inhibitor substance is present and prevents agglutination.

methods will demonstrate antibodies in as many cases as does the slide test. Indeed, investigators carefully performing sensitive tube tests have reported the demonstration of anti-Rh agglutinins in sera of women bearing erythroblastotic babies in a higher percentage of cases than have workers using the incubation method outlined above.[6, 7] The "blocking test" will probably also demonstrate antibodies in a higher percentage of cases of sensitized women if the variables are so arranged as to favor inhibition. This would very likely involve the use of cells and sera of several specificities, or careful titration to demonstrate zoning, which also apparently indicates inhibitor activity. Since such procedures are laborious, the simplicity of the slide test recommends it for clinical use.

By the slide test, anti-Rh agglutinins have also been demonstrated in the sera of nine Rh-negative persons known to have been sensitized by transfusion of Rh-positive blood in whom the incubation method gave negative results.

This method has not yet received adequate trial to be proposed as a substitute for the incubation method of demonstrating anti-Rh agglutinins. Indeed, although cells of different specificity frequently react a little differently with the various sera, the reactions are usually not sufficiently clear cut on the slide for distinguishing the subgroups of Rh-positive cells. Moreover, it is essential to determine the clinical importance of the inhibitor substance, and for this purpose it seems necessary that parallel tests by the three different methods continue to be performed. For clinical work, however, the slide test does appear to possess the following advantages:

1. It appears to demonstrate Rh sensitization in a higher percentage of cases than does any other single test.
2. It more nearly pictures what is likely to occur in vivo, where a relatively large amount of agglutinogen is ordinarily exposed to a smaller amount of agglutinin, both in cases of incompatible transfusion and in cases of erythroblastosis fetalis.
3. It is quickly performed with simple equipment.
4. It permits the use of testing sera heretofore considered of no value.

SUMMARY AND CONCLUSIONS

A simple and effective slide test is described for the demonstration of anti-Rh agglutinins in the sera of sensitized individuals, and the advantages of this method are outlined. In contrast to the incubation test, which fails to show the presence of anti-Rh agglutinins in over 50 per cent of cases chiefly because of the presence of inhibitor substances, this slide test has demonstrated agglutinins in the sera of seventy-nine of eighty women who have delivered infants with proved erythroblastosis fetalis and in nine persons who had been sensitized by transfusions of Rh-positive blood cells.

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THE BASIC ELECTROCARDIOGRAPHIC PATTERNS IN BUNDLE BRANCH BLOCK

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IN A recent paper we studied the distribution of electrocardiographic potentials over the surface of the body in the normal and hypertrophied heart.[1] Using a similar method, we have extended our observations to cases of bundle branch block in an endeavor to correlate the electrocardiographic patterns observed with the actual spread of the impulse through the ventricles.

The concept of bundle branch block was first developed by Eppinger and Rothberger in 1909.[2] A few years later Carter[3] formulated the criteria for the diagnosis of bundle branch block in man. It was recognized at that time that there were two main types of bundle branch block patterns in the standard leads. The first type consisted of records in which the main QRS deflection of Lead I was upright. This was interpreted as right bundle branch block by Lewis and others.[4] The second type consisted of records in which QRS-1 pointed downward. This was interpreted as left bundle branch block.

The first serious objection to this classification came from the work of Barker, Macleod, and Alexander[5] and from the observations of Wilson and his associates,[6, 7] who used unipolar and multiple precordial leads in their studies. They showed that cases of so-called right bundle branch block were actually due to disturbance of the main left bundle and that cases of so-called left bundle branch block were in reality due to interruption of the left main bundle. These concepts of Wilson are generally accepted today.

In the observations described in this paper we have accepted and extended the principles of bundle branch block enunciated by Wilson. Although we have made use of unipolar leads, my modification[8] of Wilson's indifferent electrode of zero potential[9] was used in taking the unipolar leads, and the unipolar extremity leads were taken with our technique of obtaining augmented unipolar extremity leads.[8] The records obtained, however, are identical with those taken according to the original technique of Wilson.[9]

I. MATERIAL

For the purposes of this study we made use of a series of electrocardiograms from fifty cases of bundle branch block drawn from our files. In all these cases we had taken not only the standard leads, but also unipolar extremity leads and multiple unipolar precordial leads, using the technique mentioned.

Further, in twenty-five of these cases multiple unipolar precordial leads had also been taken from the following points on the surface of the body:

2 R.ics.—Electrode on right mid-clavicular line at level of second intercostal space

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2 m.st.—Electrode on sternum at junction of manubrium and body of sternum

2 L.ics.—Like 2 R.ics. but on left mid-clavicular line

4 R.ics.—Electrode on right mid-clavicular line at level of fourth intercostal space

R.U.A.—Electrode on right upper abdominal wall on continuation of right mid-clavicular line, at level of lowest border of ribs

L.u.B.—Electrode on left upper back at the base of spine of left scapula

L.m.B.—Electrode at angle of left scapula

L.l.B.—Electrode on left lower back on twelfth intercostal space, directly below angle of left scapula

R.u.B.—Like L.u.B. but on right side

R.m.B.—Like L.m.B. but on right side

R.l.B.—Like L.l.B. but on right side

The multiple unipolar precordial leads were taken in the following way:

V-1—Electrode at right sternal border on fourth intercostal space

V-2—Electrode at left sternal border on fourth intercostal space

V-3—Electrode placed midway on line drawn from the points of application of Leads V-2 and V-4

V-4—Electrode on left mid-clavicular line at level of fifth intercostal space

V-5—Electrode on left anterior axillary line at level of fifth intercostal space

V-6—Electrode on left mid-axillary line at level of fifth intercostal space.

All cases in which myocardial infarction was present in addition to the bundle branch block were excluded from this series.

We also studied electrocardiograms from five cases of left ventricular hypertrophy in which there was a prolongation of the QRS interval (0.11 second or more), using the above leads.

II. PHYSIOLOGIC CONSIDERATIONS

When heart muscle becomes activated, electrical potentials are produced, the active muscle being relatively (−) to the inactive muscle (and the inactive muscle being relatively [+] to the active). This potential difference exists at the junction between the inactive muscle and the muscle which is undergoing activation or regression, because muscle which is fully active (or inactive) has the same potential throughout, and such a region can have no effect on the electrocardiogram. As the impulse travels over the heart muscle, it may therefore be regarded as having a (+) pole in the direction toward which it is progressing and a (−) pole in the direction from which it is passing (Fig. 1). If impulses from two or more regions of the heart are traveling in the same direction, their effect will be augmented. If they are traveling in opposite directions, they will tend to counterbalance each other.

In the normal heart, the impulse travels from the subendocardial region outward to the epicardium. Theoretically both sides of the interventricular

septum are considered to be activated simultaneously,[10] although this probably is not so.[11, 12] (Whether it is or not is not important to the thesis of this paper.) However, in a case in which one of the main branches of the bundle of His is interrupted, one side of the septum is definitely activated in advance of the other, and from a theoretical point of view the electrocardiographic patterns which would be obtained from a unipolar lead overlying the surface of the left ventricle and facing the left side of the septum and from a unipolar lead overlying the surface of the right ventricle and facing the right side of the septum can be anticipated from a knowledge of the spread of the impulse. For example, in a case in which conduction through the right main branch of the bundle of His has ceased for one reason or another, the following conditions would exist *(right bundle branch block):*

1. The first region of the ventricle to be activated in such a case is the left side of the septum, and the impulse will begin to spread through the septum toward the right side. The effect of this initial electrical activity on the unipolar leads would be to cause a (+) upward deflection in the lead facing the right side of the septum and a (−) downward deflection from the left ventricular lead (Fig. 1, *a*).

2. Following this, the impulse travels outward through the normally activated left ventricle and at the same time completely penetrates the septum to reach its affected right ventricular surface. With the activation of the right side of the septum, the entire septum becomes fully activated and, as mentioned previously, the potential differences formerly present disappear and the deflection should return to the isoelectric line. However, since at the same time there is an impulse passing outward through the left ventricular wall, the right ventricular electrode faces the tail end of this wave and a (−) deflection is recorded instead. The impulse which travels outward through the left ventricle also serves to limit the depth of the initial downward deflection recorded by the left ventricular electrode. The left ventricular electrode, facing the oncoming wave through the left ventricle, records a (+) deflection (Fig. 1, *b*).

3. Once the impulse has penetrated the septum it spreads to that portion of the affected conducting system still functioning and then outward through the wall of the affected right ventricle. The potential at the left ventricular electrode (which had been recording a [+] deflection) tends to become isoelectric when the activation of the left ventricle is complete and the surface of the left ventricle is reached. Since, however, the right ventricle is still being activated, the left ventricular electrode faces the tail end of this wave, passing outward through the right ventricle, and the deflection becomes (−). The right ventricular electrode faces the oncoming wave in the right ventricle and records a (+) deflection (Fig. 1, *c*).

4. The last region of the heart to become activated is the right ventricular surface. When this occurs the (+) potential at the right ventricular electrode becomes isoelectric, as does the (−) deflection of the left ventricular electrode (Fig. 1, *d*).

With left bundle branch block the potentials of the right and left ventricular leads should be just the reverse (Fig. 2).

It can therefore be said that an electrode overlying the affected ventricle and facing the affected side of the interventricular septum should record an M-shaped QRS complex, and an electrode overlying the contralateral normal ventricle and facing the normal side of the septum should record a W-shaped QRS complex. Furthermore, inasmuch as conduction through the muscle of

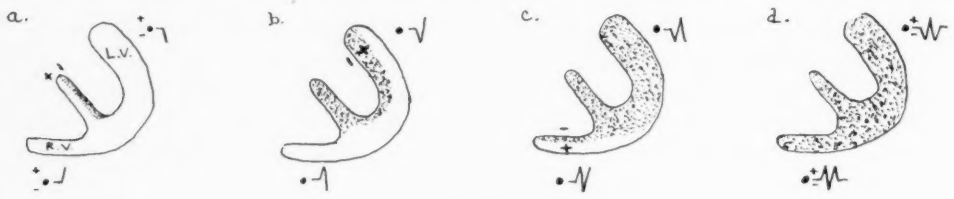

Fig. 1.

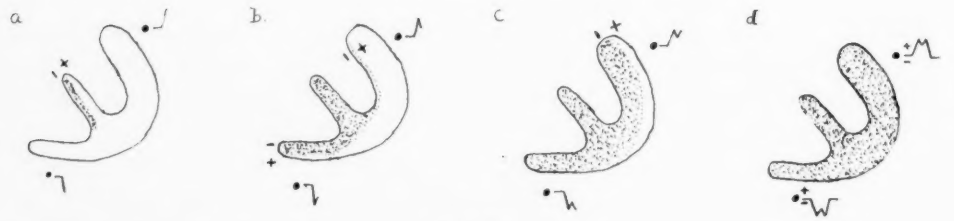

Fig. 2.

Figs. 1 and 2.—Schematic representation of the spread of the impulse through the heart in cases of bundle branch block and the basic electrocardiographic patterns which would be obtained. The stippled areas represent regions which are being or have been activated. The heavy dots represent electrodes, and the graphs next to them represent unipolar electrocardiograms which theoretically would be obtained if the heart were in the stage of activity represented in the drawings. For further details see text.

the interventricular septum is slower than through the conduction system,[13] it would be expected that the duration of the QRS complex, which is a measure of this, should be increased. That this actually occurs can be seen in the following examples:

1. Fig. 3 is the record of a case of right bundle branch block. Note the M-shaped QRS in the precordial Leads V-1 V-2, and V-3, which face the affected right side of the septum, and the W-shaped QRS in the precordial Leads V-4, V-5, and V-6, which face the normal left side of the septum.

2. Fig. 4 is the record of another case of right bundle branch block. Note the M-shaped complexes in precordial Leads V-1 and V-2 and in the unipolar leads from the second right intercostal space and the right mid-back. The W-shaped QRS can be seen in precordial Leads V-5 and V-6 and in the lead from the left mid-back, although the initial downward deflection in the precordial leads is small in this case.

3. Fig. 6 is that of a case of left bundle branch block. Note that here the M-shaped QRS is found in the leads facing the affected left ventricular side of the septum, namely, precordial Leads V-5 and V-6 and the leads from the left upper and lower back in this case. The W-shaped QRS is found, on the other hand, in those leads which face the normal right side of the septum, namely, in this case, the leads from the second intercostal space anteriorly (2 R.ics., 2 m.st., 2 L.ics.), the first precordial Lead V-1, and the right upper abdominal lead.

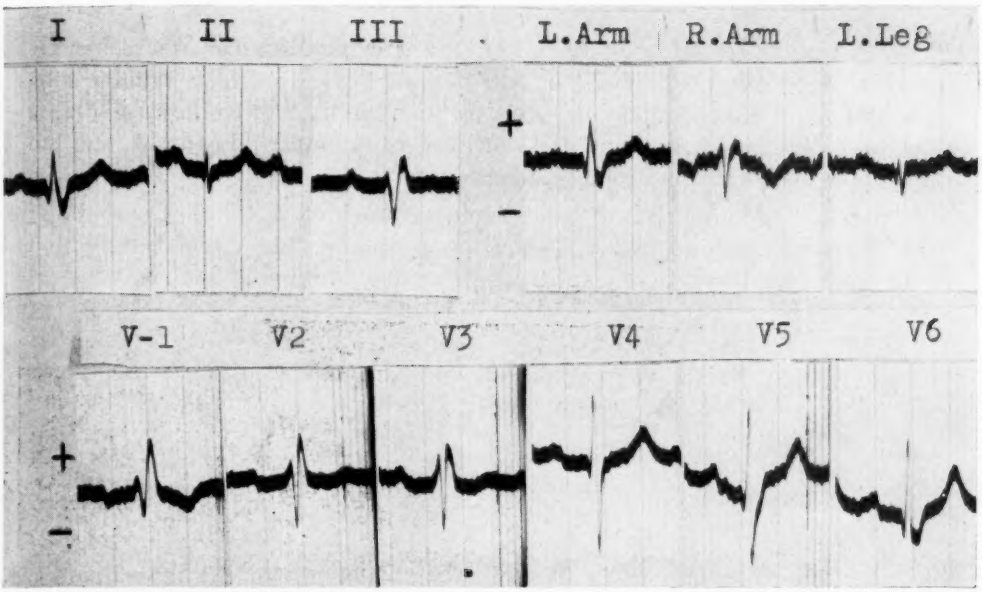

Fig. 3.—R. W., woman, 53 years of age. Right bundle branch block; heart in oblique position.

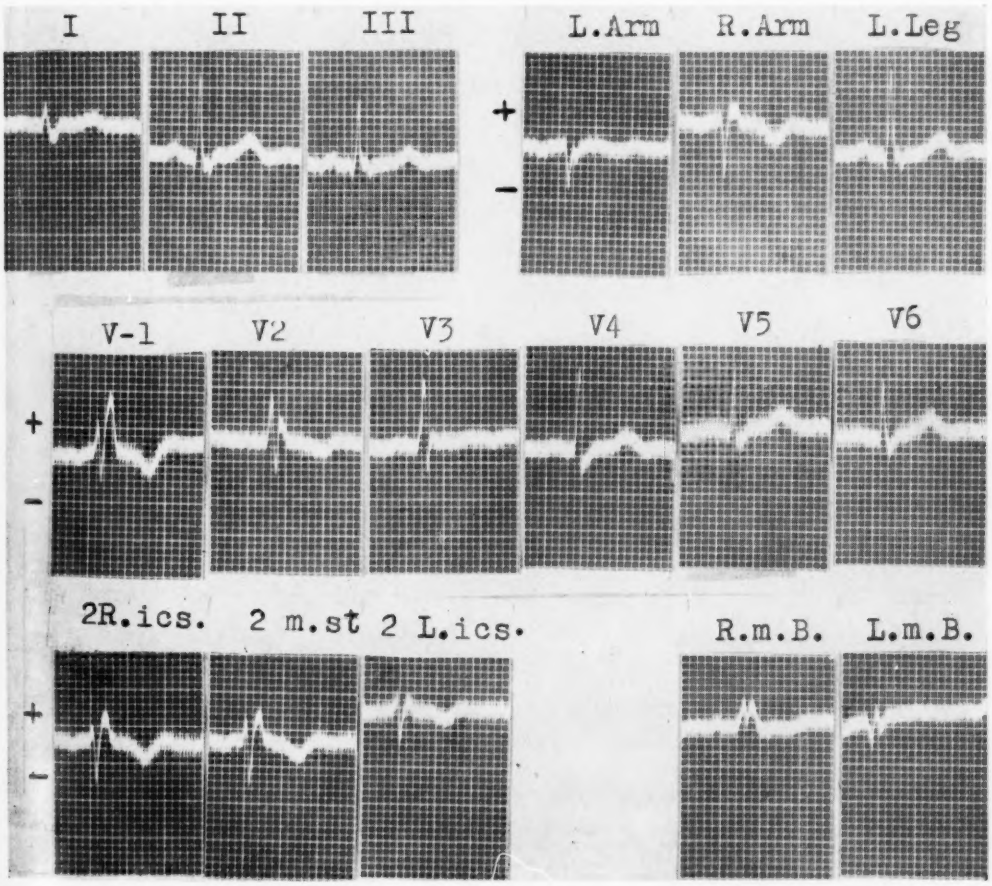

Fig. 4.—I. G., man, 85 years of age. Right bundle branch block; heart in vertical position.

4. A similar situation holds in Fig. 7 where the M-shaped complexes are found in precordial Leads V-4, V-5, and V-6 and in the lead from the left mid-back, and the W-shaped QRS complexes are found in leads from the second right interspace, the mid-sternal lead, the fourth right interspace, Lead V-1, and the right upper abdominal and right mid-back leads.

It should be noted that with left bundle branch block, the notch of the M or W does not reach or extend beyond the isoelectric line in contradistinction to the conditions theoretically anticipated and as found in cases of right bundle

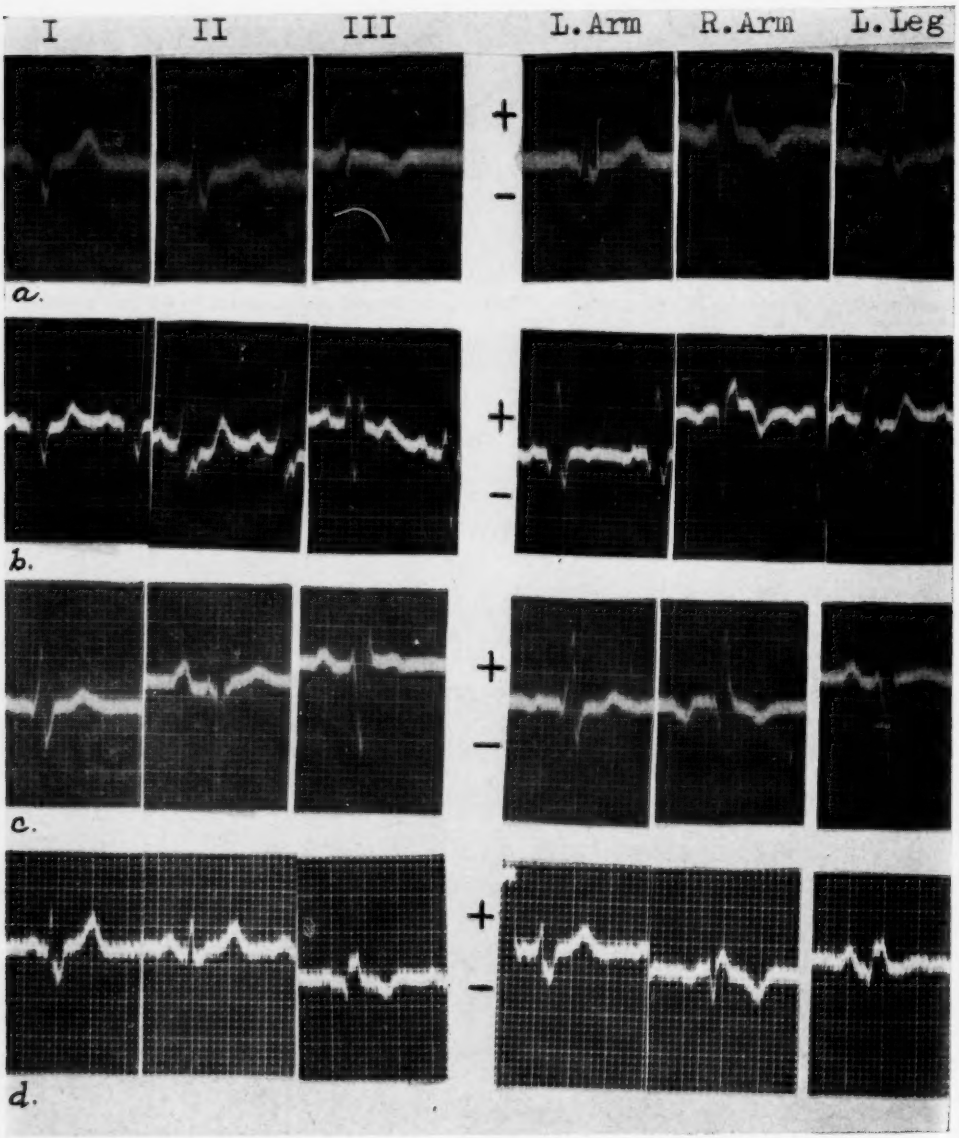

Fig. 5.—Additional cases of right bundle branch block. In *a*, *b*, and *c* the hearts are more or less in the oblique or horizontal position. In *d* the heart is more or less vertical. *a*, B. R., woman, 54 years of age; *b*, V. W., woman, 66 years of age; *c*, E. B., man, 51 years of age; *d*, R. B., woman, 67 years of age.

branch block. The explanation for this is as follows: In left bundle branch block, the initial activity of the right side of the septum is recorded as a (−) deflection at the right ventricular electrode (Fig. 2, *a*). The impulse traveling

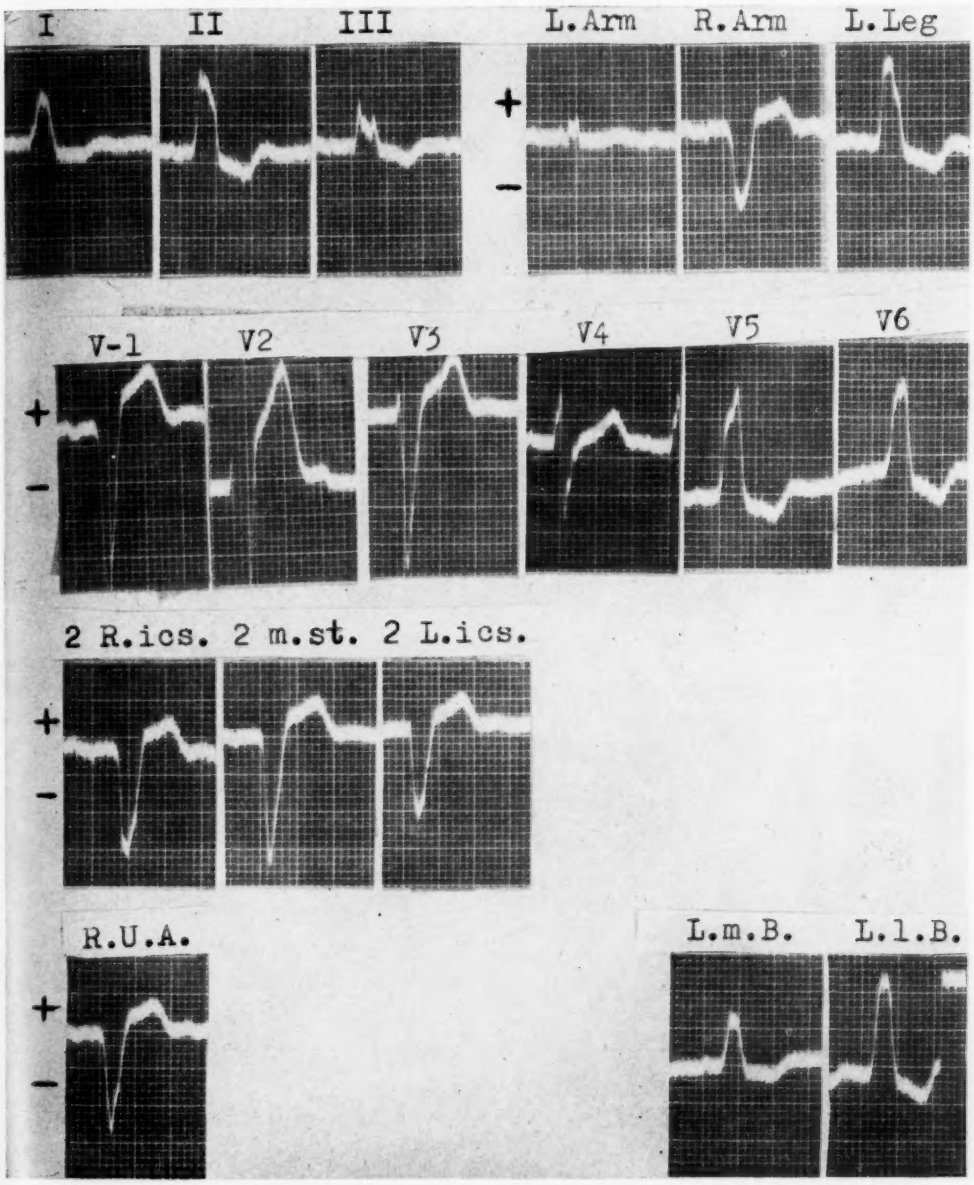

Fig. 6.—I. M., man, 88 years of age. Left bundle branch block; heart in moderately vertical position.

outward through the normal right ventricle a moment later tends to counterbalance this initial activity, but since the right ventricular wall is thinner than that of the left ventricle, the electrical activity produced within the right ventricle is weaker, and it does not interfere as much with the initial down-

ward deflection as left ventricular activity does in a case of right bundle branch block. Consequently, the depth of the deflection is greater than that observed in the left ventricular lead in a case of left bundle branch block. For the same reason, after the septum has become fully activated and the sur-

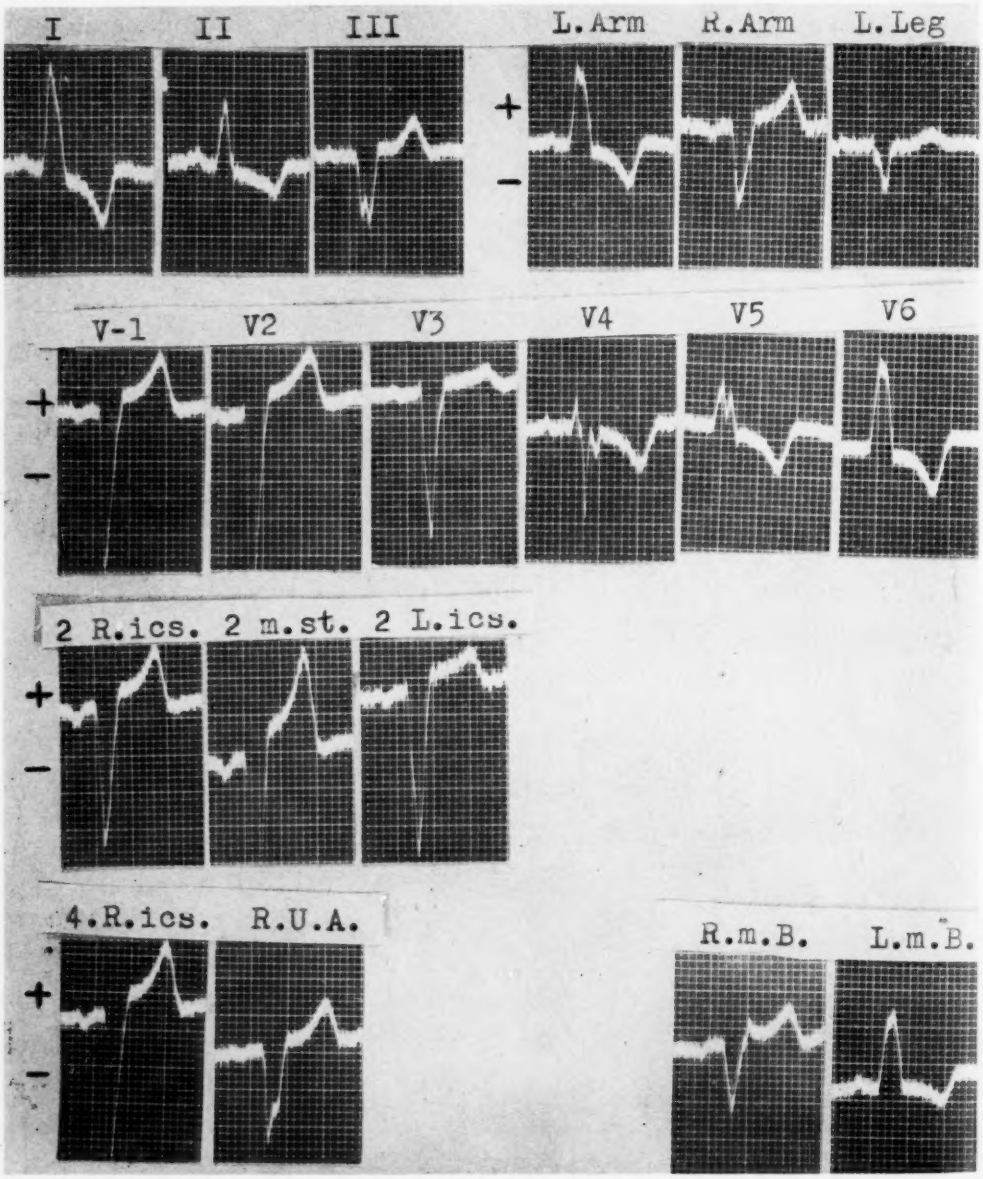

Fig. 7.—M. R., man, 63 years of age. Left bundle branch block; heart in horizontal position.

face of the right ventricle activated, the deflection tends to go to the isoelectric line, but before it can reach the isoelectric line, the impulse spreading outward through the large left ventricular wall causes the deflection to become more (−) at the right ventricular electrode (Figs. 2, *b* and *c*). The final

deflection is recorded as the surface of the affected left ventricle is activated (Fig. 2, *d*). The electrical activity recorded at the left ventricular electrode has, of course, the opposite polarity as that recorded by the right ventricular electrode.

In many cases it will be noted that instead of actual notching, which produces the M and W, the peak of QRS is just slurred. Also, QRS deflections, more complex in shape than an M or W, are occasionally observed.

III. THE PATTERNS IN STANDARD AND UNIPOLAR EXTREMITY LEADS

The relations between standard leads and unipolar extremity leads can be summarized in the following way:[1, 14] Lead I tends to resemble the left arm lead and Lead III tends to resemble the left leg lead. Since the extremities are far removed from the heart, the patterns tend to vary widely for several reasons. First, the exact area of the heart that the electrode faces is much more extensive. Second, as has been shown previously,[1] the extremities occupy transition zones of potential and vary greatly with the position of the heart. When the heart lies vertically, the left leg lead faces the left ventricle and the left side of the septum, and the left arm lead tends to face the endocardium of the ventricles. When the heart lies obliquely or horizontally, it is the left arm lead which faces the left ventricle and the left side of the septum, the left leg lead tending to face the epicardial surface of the right ventricle and the right side of the septum.

Thus in right bundle branch block, the following patterns may be theoretically anticipated in the unipolar extremity and standard leads:

1. *With a vertical heart,* the left leg lead should have a W-shaped QRS. The left arm lead, though facing the endocardium and therefore being predominantly (−),[14] also faces the left side of the septum and should also have a W-shaped QRS.

2. *With an oblique or horizontal heart,* the left arm lead should have a W-shaped QRS and the left leg lead should have an M-shaped QRS.

3. Irrespective of the position of the heart, the right arm lead tends to face the right side of the septum and should have an M-shaped QRS.

Actual unipolar extremity leads in cases of right bundle branch block correspond well to this. Fig. 4 illustrates the records in a vertical heart. Fig. 5, *a, b, c,* are cases in which the heart is more or less horizontal.

Occasionally with right bundle branch block occurring in a vertical heart, the left arm lead will tend to face the right side of the septum. The reason for this is that with a vertical heart, there is also rotation of the heart around its long axis, which causes the right ventricle to become more anterior and the apex to move posteriorly. In such a case the left arm lead will tend to have an M-shaped QRS (Fig. 5, *d*). Note that Lead I tends to resemble the left arm lead and that Lead III tends to resemble the left leg lead.[1]

The position of the heart is also probably responsible for the variations found in the unipolar precordial leads. For example, in Figs. 3 and 4, Leads V-1, V-2, and V-3 have an M-shaped QRS, although other of our cases showed the M-shaped QRS in precordial Leads V-1, or Leads V-1 and V-2, or even in Leads V-1, V-2, V-3 and V-4.

It should be noted that the right arm lead often does not show an initial upward deflection. In such cases, leads from the right upper chest wall anteriorly will have the characteristic deflection. This is emphasized because in left bundle branch block neither the right arm lead nor leads from the right upper anterior chest wall will have upward initial deflections.

It may also be noted that we not infrequently find records which are typical of right bundle branch block with the exception of the fact that the QRS interval does not exceed 0.1 second. The significance of such records, which we have found in normal subjects, will be discussed elsewhere.[11]

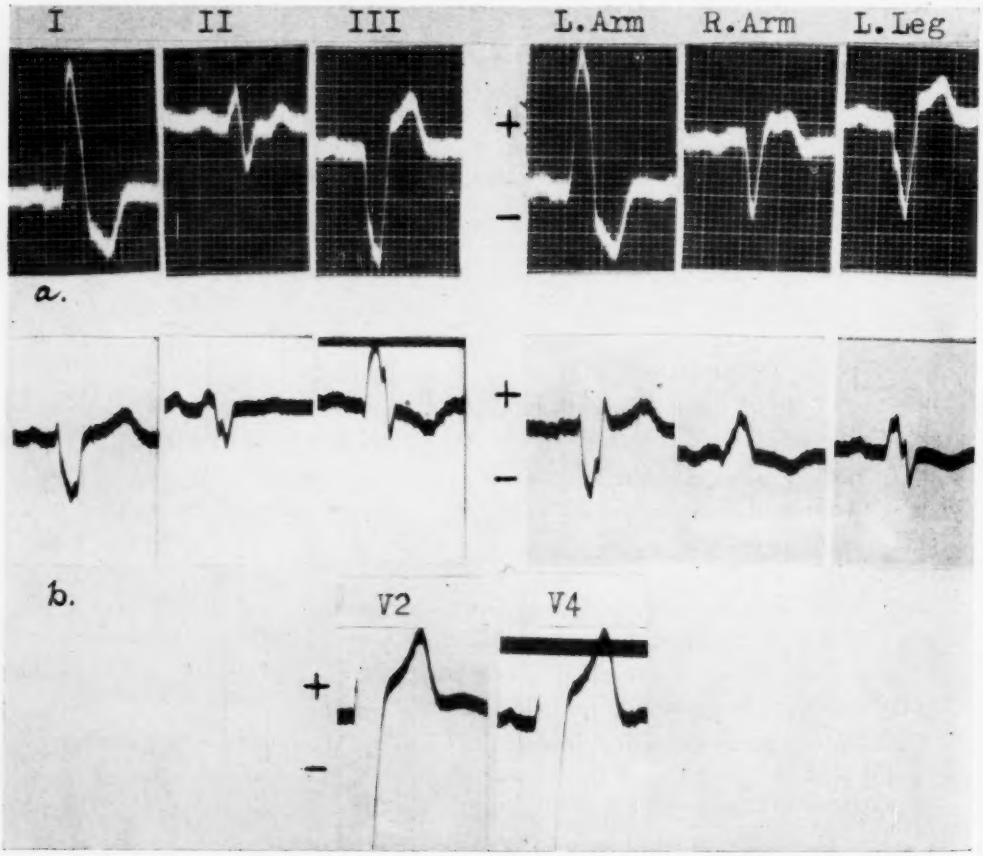

Fig. 8.—Additional cases of left bundle branch block. *a*, P. A., man, 59 years of age; heart in horizontal position. *b*, M. W., woman, 62 years of age; heart in vertical position.

In left bundle branch block *with an oblique or horizontal heart*, the left arm lead faces the left ventricle and the left side of the septum and should have an M-shaped QRS. In such a case the left leg lead tends to face the right ventricular surface and the right side of the septum and should have a W-shaped QRS. *A vertical heart* is uncommon in left bundle branch block because most of the cases have enlargement of the left ventricle which is the cause of the horizontal position of the heart. When left bundle branch block does occur with a vertical heart, however, the left leg lead will face the left side of the septum and should have an M-shaped QRS; the left arm lead

should be small but should also have an M-shaped QRS. Irrespective of the position of the heart, the right arm lead and leads from the right upper chest anteriorly should always have an initial downward deflection. This actually occurs. Fig. 6 is that of a moderately vertical heart; Figs. 7 and 8, *a*, of horizontal hearts with left bundle branch block. Note that in Fig. 7 the initial deflection of the left leg lead is upward instead of downward, as theoretically anticipated. The explanation of this is that the left leg lead does not face the right ventricle sufficiently to record the initial (–) deflection from the right side of the septum. In such a case leads farther to the right, as from the

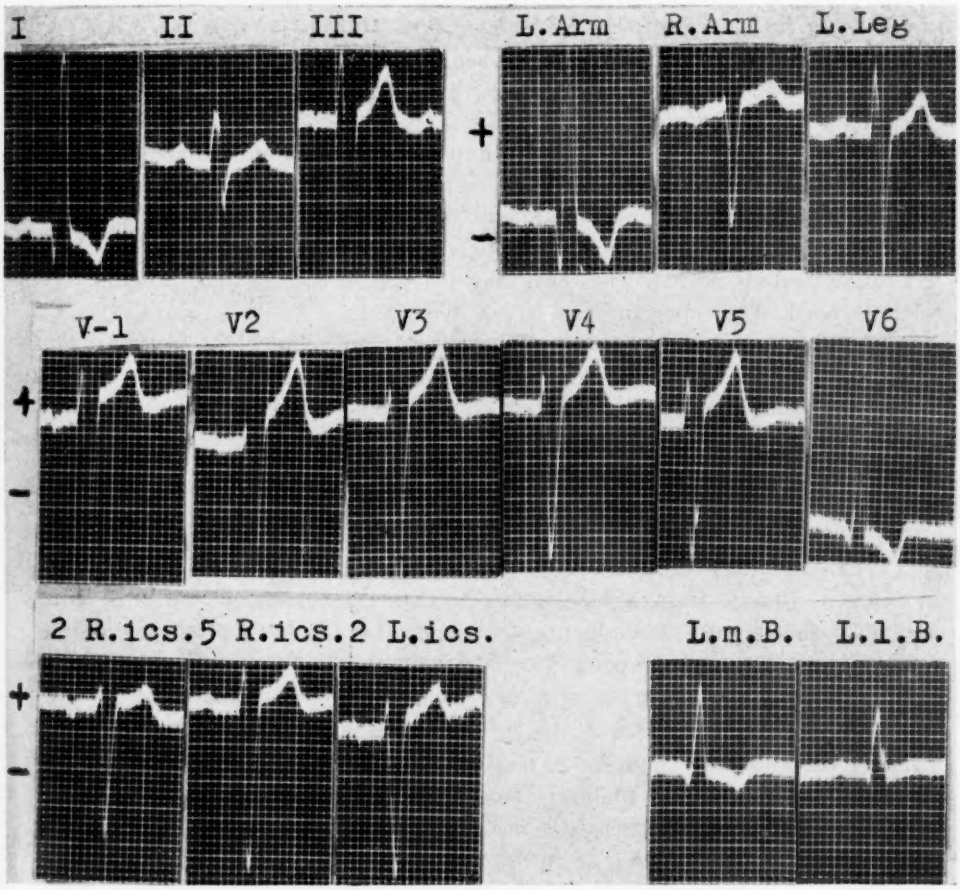

Fig. 9.—A case of left ventricular hypertrophy with prolongation of the QRS interval. J. E., man, 24 years of age; rheumatic heart disease and aortic insufficiency.

right back or right abdominal wall, often do have the initial downward deflection (Fig. 7). In Fig. 8, *a*, the initial downward deflection in the left leg lead is present. Fig. 8, *b*, is a record of a case of left bundle branch block in which the heart was markedly vertical. A similar record has been published by Wilson.[10]

Variations, also probably due to the position of the heart, are found in the precordial leads in cases of left bundle branch block. Usually, though not necessarily, the first precordial lead, V-1, has an initial downward deflection,

although this may also be seen in Lead V-2. The M-shaped QRS is usually not seen until the electrode is moved sufficiently to the left, usually to point V-5.

In addition to our studies on cases of bundle branch block we took multiple unipolar leads over the surface of the body in five cases of hypertensive cardiovascular disease and left ventricular hypertrophy. In these particular cases the electrocardiogram showed a prolongation of the QRS interval to either 0.11 second or more. Fig. 9 illustrates such a case, that of a 24-year-old man with rheumatic heart disease, aortic insufficiency, and massive enlargement of the left ventricle. Note that although the pattern superficially resembles that of left bundle branch block, leads from the right upper portion of the body have an initial upward deflection, and leads from the left side of the body have an initial downward deflection. The pattern is therefore similar to that of a normal horizontal heart[1] or to cases of right bundle branch block, insofar as the initial deflection is concerned, rather than left bundle branch block. The characteristic M- and W-shaped patterns of bundle branch are not seen. The prolonged QRS interval in such a case can be explained by the massive size of the patient's heart and the fact that it takes the impulse a longer time to penetrate the hypertrophied left ventricle than a normal heart. A similar prolongation of the QRS may be seen in cases where the right ventricle is markedly hypertrophied.

The T wave patterns observed in cases of left and right bundle branch block should be discussed briefly. An M-shaped QRS usually has a (−) T and W-shaped QRS an upward (+) T, although exceptions to this are common. These T wave patterns are due to the altered paths over which the impulse spreads and are not due to myocardial damage.[10]

IV. CONCLUSIONS

When unipolar leads are used, the electrocardiographic patterns observed in cases of bundle branch block may be directly correlated with the actual spread of the impulse through the ventricles. On the basis of theoretical considerations and actual electrocardiographic observations, the following are the basic electrocardiographic patterns in cases of bundle branch block:

1. An M-shaped QRS complex in unipolar leads overlying or facing the affected ventricle and the affected side of the interventricular septum.

2. A W-shaped QRS complex from unipolar leads overlying or facing the contralateral normal ventricle and the normal side of the septum.

3. Prolongation of the QRS interval to 0.11 second or longer.

4. A (−) T wave with the M-shaped QRS and a (+) T with the W-shaped QRS, although exceptions to this are frequent.

5. The patterns observed with unipolar extremity and standard leads depend on the position of the heart. When the heart is vertical, the left leg lead will face the left ventricle and the left side of the septum; when the heart is oblique or horizontal, the left arm lead will face the left ventricle and left side of the septum, the left leg lead now tending to face the right ventricle and the right side of the septum.

In this paper we also demonstrated the fact that *not* every case in which the QRS interval exceeds 0.11 second is necessarily due to bundle branch block but may be explained by the fact that the impulse requires a longer time to penetrate a greatly hypertrophied ventricle than it normally needs.

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THE PRODUCTION OF BACTERICIDAL ACTIVITY IN COD-LIVER OIL BY OXIDATION

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THE Council on Pharmacy and Chemistry of the American Medical Association, in 1943,[1] conducted a thorough review of the literature appertaining to the value of cod-liver oil as a medicinal agent promoting the healing of wounds following topical application. Most of the controversy centered about the virtures of the agent depending upon the vitamin content of the oil. The Council concluded, at that time, that the evidence available did not warrant the acceptance of cod-liver oil preparations for the topical treatment of wounds.

Reports by Harris, Bunker, and Milas (1932)[2, 3] Löhr and Treusch (1934),[4] Stevens (1936),[5] Nélis (1939),[6] Lichtenstein (1939),[7] and Worley (1940)[8] indicate that cod-liver oil elicits bactericidal activity under various conditions. However, Kummell and Jensen (1936)[9] and Driver, Binkley, and Sullivan (1938)[10] failed to observe bactericidal activity in their preparations. These contradictory data have led us to investigate this aspect of the question. After evaluating the data available, it appears that so-called old or rancid cod-liver oils are usually bactericidal. Furthermore, treatment by heat, aeration, or ultraviolet irradiation in shallow, open dishes results in a preparation possessing bactericidal activity. Fresh cod-liver oils are rarely bactericidal. These observations suggest that the substances lethal to microorganisms are produced by the interaction of the oil with oxygen. While this was undoubtedly appreciated by earlier workers, the importance of the initial oxidation has not been recognized as the basic reaction leading to the synthesis of substances possessing bactericidal activity.

According to Hilditch (1941)[11] 85 to 89 per cent of cod-liver oil consists of unsaturated fats. Such of these fats as contain the ethylene linkage arranged in the highly reactive 1-4 conjugated system of double bonds, — CH=CH—CH=CH—, are known to undergo autoxidation by atmospheric oxygen and may initiate chain reactions.

The presence of naturally occurring antioxidants (Olcott and Mattill 1941[12]) in fats retards and may inhibit the oxidation of the unsaturated fats. Buxton (1942)[13] further regarded the presence or absence of antioxidants as factors in the destruction of vitamin A. Buxton's procedure for the removal of these antioxidants from cod-liver oil has been followed in this study. The development of bactericidal properties by these oils subsequent to oxidation by atmospheric oxygen is studied in this investigation.

Materials and Methods.—Commercial U. S. P. cod-liver oil was used in these experiments. After adding one part of a nonpolar solvent such as benzene,

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chloroform, or hexane to two or three parts of oil, five parts of this mixture were treated by stirring with one part of activated Norit for thirty minutes to an hour at room temperature. Following filtration, the solvent was distilled off at reduced pressure. This treatment removed the antioxidants, and the residual oil readily underwent oxidation when exposed to atmospheric oxygen.

The rate of production of perioxides and aldehydes was determined by the methods of Lea (1931).[13a]

The bacteriologic tests were made using plates of infusion agar heavily inoculated with *Staphylococcus aureus*, *Bacillus subtilis*, or *Escherichia coli*. The oils were tested by direct contact on the agar using filter paper disks. The

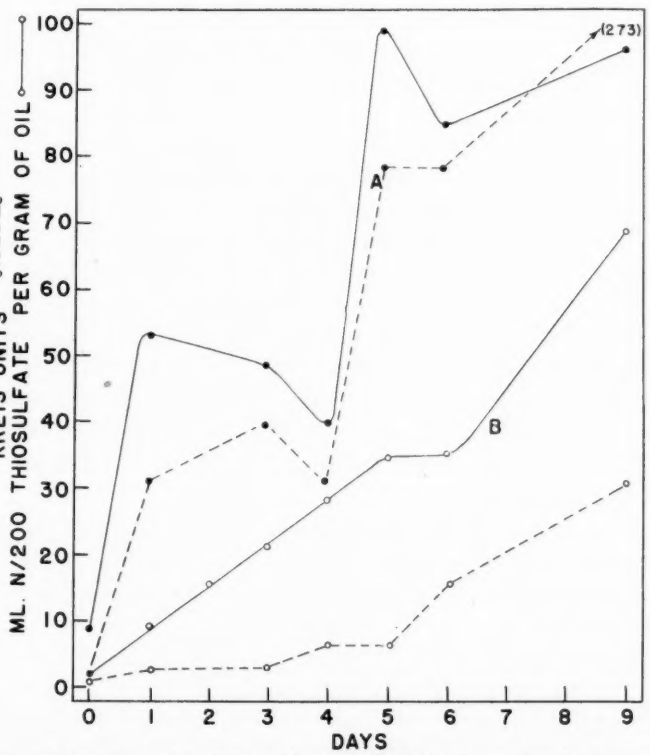

Fig. 1.—Illustrating the rate of peroxide and aldehyde formation in (*B*) a specimen of U. S. P. cod-liver oil and in (*A*) the same oil after extraction with activated Norit to remove the antioxidants in commercial cod-liver oil. The irregularity of these curves would indicate that the observed values are the summation of several reactions. The solid lines represent the peroxide values, while the dashed lines represent those of the aldehydes. (*A* and *B* are the specimens *A* and *B* of Table I.)

vapors of the oils were tested by pouring 5 to 8 c.c. of oil into the bottom of Petri plates and inverting a second plate bottom containing the inoculated agar over the oil. Cultures and oils were incubated at 38° C. and read at twelve and twenty-four hours.

Experimental.—When the untreated U. S. P. cod-liver oils were tested for bactericidal activity, it was found that they were not bactericidal. After extracting the antioxidants from the oils, the preparations exhibited bactericidal properties by direct contact and by the vapors emanating from the preparations following exposure to atmospheric oxygen. Dishes of the oils were exposed to

atmospheric oxygen at 38° C. and tested daily for bactericidal activity and for the formation of peroxides and aldehydes. Adsorbed oils rapidly became bactericidal and remained so indefinitely. In contrast, the fresh U. S. P. oils did not elicit bactericidal effect until they had been exposed for at least five days.

The rate of formation of peroxide is shown in Fig. 1 in a specimen of fresh U. S. P. cod-liver oil and in another specimen of the same oil from which the antioxidants had been removed. It is apparent that the rates of oxidation differ greatly in these instances. The extracted oil was bactericidal to the three organisms testing within the first twelve hours of incubation at 38° C. In contrast, the original oil oxidized comparatively slowly and did not become bactericidal until the sixth day of incubation. While the peroxide oxygen values, expressed in cubic centimeters of N/200 thiosulfate, can only be considered as semiquantitative, because of the complex reactions taking place (Milas, 1941[14]), the values do indicate large increases in peroxide and the length of time required for an oil to undergo auto-oxidation after it has been exposed to the air. In Table I is shown the increase in aldehydes in these same oil specimens. The Kreis reaction for aldehydes is essentially a qualitative test. It was modified as follows to yield relative quantitative values: the reactants produced a pink color. An ethereal solution of phloroglucinol was added to the oil dissolved in chloroform, and the mixture was acidified with HCl. The color intensity was compared with an arbitrary set of standard tubes containing varying concentrations of $KMnO_4$. Tube 1 was designated as 1 unit; Tube 2 contained 2.5 times the concentration of Tube 1, etc.; Tube 5 contained 39 Kreis units of color. As the reaction proceeded to exceed the color intensity of Standard Tube 5, the oil was diluted with chloroform and tested. The number of units was then multiplied by the proper dilution factor to obtain the relative quantity of aldehyde in the specimen.

The data presented in Fig. 1 and Table I show the rate of peroxide formation and aldehyde production to be much more rapid in the case of the oils from which the antioxidants have been extracted than in the instance of the original U. S. P. cod-liver oil.

TABLE I

THE RELATIVE QUANTITIES OF ALDEHYDES PRODUCED DURING OXIDATION OF COD-LIVER OIL AS INDICATED BY THE KREIS REACTION (A AND B ARE A AND B OF CHART 1)

TIME (DAYS)	ADSORBED OIL A (KREIS UNITS PER C.C.)	OIL B (KREIS UNITS PER C.C.)
0	2.5	1
1	31.2	2.5
3	39	2.5
4	31.2	6.25
5	78	6.25
6	78	15.6
9	273	31.25

The Effect of an Antioxidant.—A specimen of extracted cod-liver oil was divided into three portions: A, B, and C. Hydroquinone was added to C in a ratio of 1 to 1,000. The three specimens were aerated with oxygen at 80° C. and the rate of peroxide formation determined. The rate of peroxide formation was retarded in C. After two and one-half hours, hydroquinone was added to B in a ratio of 1 to 1,000. The addition of the antioxidant to specimen B did not

alter the rate of peroxide formation once the reaction was underway (Fig. 2). The rate of aldehyde production was determined and was found to be comparable with that observed in Fig. 1. No aldehyde was formed in specimen C. After heating for four hours, the three specimens were tested for bactericidal activity. A and B were strongly bactericidal. C exhibited no bactericidal activity.

The Chemical Nature of the Bactericidal Substance.—Since the bactericidal effect was present both in the oil and in the vapors given off by the oil, it was evident that at least one bactericidal substance formed had a low boiling point. Testing these vapors with acidified starch-iodide and Schiff's reagent showed

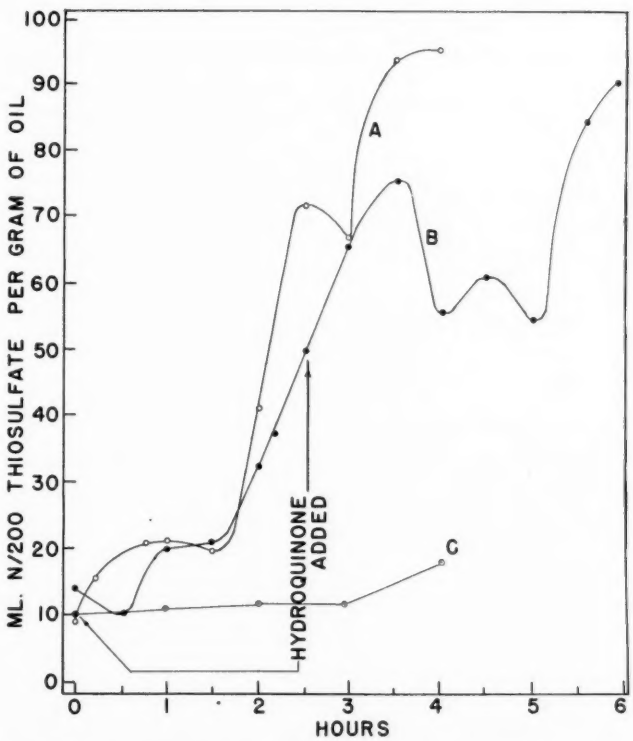

Fig. 2.—A lot of extracted cod-liver oil was divided into three portions, *A*, *B*, and *C*, and heated to 80° C. while oxygen was bubbled through each specimen.

Hydroquinone, an antioxidant, was added to *C* in a ratio of 1 to 1,000 before heating. After heating for 2½ hours, hydroquinone was added to *B* in the same proportions. *C* did not undergo oxidation, while *A* and *B* oxidized rapidly. Once oxidation had begun, the reaction was not suppressed by the antioxidant, Curve *B*.

them to contain both peroxides and aldehydes. Vapors from fresh U. S. P. cod-liver oil were not bactericidal and failed to give positive tests for either aldehydes or peroxides. After exposing fresh U. S. P. cod-liver oil to atmospheric oxygen for at least five days, the vapors became bactericidal and gave positive reactions for both peroxides and aldehydes. Specimens of extracted oils became dark in color and viscous when incubated at 38° C. for several months. The oils and their vapors were strongly bactericidal. The oils gave positive tests for both aldehydes and peroxides; the vapors, however, gave positive reactions for aldehydes only. These vapors contained no oxidizing substances, but they continued to have bactericidal properties and possessed an acrid odor resembling

acrolein. It has not been possible to collect enough of the volatile aldehyde for chemical identification.

If the oil was heated on an open Bunsen flame, a volatile distillate could be condensed at freezing bath temperatures. These distillates were fractionated in a Vigreaux column, and a clear, colorless liquid boiling at 52° C. was collected. This substance reduced bromine water and $KmnO_4$, was strongly bactericidal, gave a positive Schiff's test for aldehyde, and reacted with dinitrophenylhydrazine to give a compound melting at 165° C. This compound gave a mixed melting point of 165° C. with the dinitrophenylhydrazone of acrolein to indicate definitely that the distillate isolated from the heated cod-liver oil was acrolein. Obviously, it does not follow that the aldehyde occurring in the vapors of cod-liver

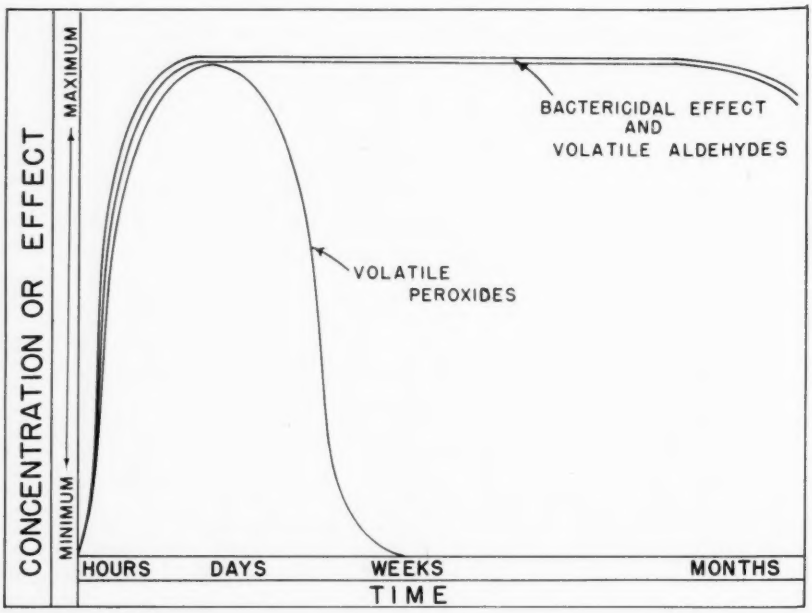

Fig. 3.—A graphic representation of the rapid and persistent production of volatile aldehydes and the acquisition of bactericidal activity by the vapors over cod-liver oil which has been treated with activated Norit to remove antioxidants. During the first days and weeks the vapors contained peroxides, but these substances ultimately disappeared although the bactericidal activity persisted, and the vapors continued to give a positive reaction for aldehydes.

oil when exposed to oxygen at 38° C. is acrolein, since acrolein results from the pyrolysis of fats in general (Egloff and Morrell, 1932[15] and Vernon and Ross, 1936[16]). The bactericidal activity of acrolein was demonstrated by Koch and Fuchs as early as 1899[17] and again by Berthelot (1922)[18] and Vollrath and coworkers (1937).[19]

DISCUSSION

The results of this investigation would indicate that the bactericidal activity acquired by cod-liver oil is dependent upon auto-oxidation. The exact chemical nature of the bactericidal substance or substances has not been determined, but one of these substances is an aldehyde and observations indicate that the substance may be acrolein. Servias (1903)[20] and Dunlap and Shenk (1903)[21] have observed volatile aldehydes with an acrolein-like odor formed in rancid cod-liver

and linseed oils. Salway (1916)[22] isolated and identified acrolein upon oxidation of linseed oil and linolenic acid. Certain volatile, saturated aldehydes do, however, likewise possess bactericidal activity (Tilley and Schaffer, 1926[23]). Harris and associates (1932),[2, 3] Stevens (1936),[5] and Lichtenstein (1939)[7] suggested that the bactericidal activity resided in the volatile peroxides found during the oxidation of cod-liver oil. Evidence presented by us shows volatile aldehydes to be present whenever the vapors over cod-liver oil possess bactericidal activity. Peroxides, however, cannot always be demonstrated in these vapors (see Fig. 3). Nélis and Thomas (1939)[6] and Worley (1940)[8] suggest that the bactericidal agents occurring in cod-liver oil are probably aldehydes.

Cod-liver oil is an effective mechanical covering for superficial lesions. Since an oil from which the antioxidants have been adsorbed produces bactericidal substances rapidly, this material suggests itself as a unique medicinal in the topical treatment of superficial wounds. This aspect of the problem is being studied especially in regard to burns and granulating surfaces. The report of the Council of Pharmacy and Chemistry of the American Medical Association records no untoward reactions following the topical use of cod-liver oil.

It is doubtful that the vitamin content of an oil contributes to its bactericidal activity. The volatile substances in the vapors from cod-liver oils would indicate that these substances have a low molecular weight.

SUMMARY

Fresh U. S. P. cod-liver oil has no bactericidal activity. Cod-liver oil acquires bactericidal activity during oxidation. This process is accelerated when the natural antioxidants in the oils are adsorbed by Norit and can be inhibited by the addition of the antioxidant, hydroquinone. Aldehydes are always present in the vapors given off by cod-liver oil when these vapors acquire bactericidal activity. It is suggested that acrolein, a bactericidal substance, may be produced by the auto-oxidation of cod-liver oil.

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THE SEROLOGIC DIAGNOSIS OF ENDEMIC TYPHUS

I. The Use of Specially Prepared Rickettsial Suspensions and Commercial Typhus Vaccines as Antigens in the Complement Fixation Test

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COMPLEMENT fixation in typhus was investigated by Castaneda,[1] who showed in 1936 that sera from human cases of the Mexican disease and from guinea pigs infected with the European strain of virus contained complement-fixing antibodies. Confirmation and extension of these observations were reported in 1941 by Bengtson,[2] who approached the problem with a view to determining the diagnostic value of the test. In this study sera from human cases of endemic typhus and from guinea pigs recovered from infections with endemic typhus were employed; the specificity of the reaction was determined with sera from cases of Rocky Mountain spotted fever and "Q" fever; normal human and guinea pig sera were used as controls. More recently, other workers, among them Reynolds and Pollard,[3] have reported on the utility and specificity of the test as a diagnostic procedure.

Antigens, in the case of Bengtson's work, were specially prepared from the inoculated yolk sac of the developing chick embryo following the method of Cox[4] and from the lungs of mice infected intranasally by the technique of Castaneda.[5] Reynolds and Pollard, on the other hand, used commercially prepared epidemic typhus vaccine as antigen. This vaccine was prepared from infected chick embryos and represented the product of a single manufacturer.

Purpose of This Study.—The purpose of this study was to determine the relative value of specially prepared rickettsial suspensions and commercially processed typhus vaccines, from a variety of sources, as antigens in the complement fixation test for typhus. At the outset, it was recognized that specially prepared antigens should prove satisfactory, but the fact that most laboratories were not in position to produce these for their own use and that they were unobtainable from commercial sources gave point to the investigation of vaccines as possible antigens as these were available on the open market.

Materials.—Specially prepared antigens made from the endemic type virus were furnished by the National Institute of Health‡ and Sharp and Dohme;§ the latter also provided a similar antigen of the epidemic type. The vaccines used were all prepared from the epidemic type virus and were manufactured by Lederle Laboratories, Inc.,‖ Parke, Davis & Co.,¶ and Eli Lilly and Co. The standard serum used in titrating the antigens and vaccines was obtained from guinea pigs which had recovered from infections with endemic typhus or from active human cases of the disease.

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§By Dr. Florence Fitzpatrick, through the courtesy of Dr. B. Hampil.
‖By Dr. H. E. Cox.
¶By Dr. F. D. Stimpert.

The Test.—The classical hemolytic system of sheep cells, guinea pig complement, and rabbit anti-sheep cell amboceptor was employed. In determining the antigenic titers of the specially prepared rickettsial suspensions or the vaccines, dilutions were first made covering the range 1:4 to 1:128. This was done by adding 0.1 c.c. of antigen to 0.3 c.c. saline in the first tube and transferring 0.2 c.c. of this dilution to the next tube which already contained 0.2 c.c. of saline. This was repeated through the remaining tubes of the series with 0.2 c.c. being discarded from the final tube. To all tubes, 0.2 c.c. of standard serum (diluted 1:8) was then added, followed by 0.2 c.c. of complement (2 units). The tubes were next incubated one hour at 37° C., after which 0.4 c.c. sensitized sheep cells was added. The sensitized sheep cell suspension was made by combining equal amounts of a 2 per cent sheep cell suspension and rabbit anti-sheep cell amboceptor which had been titrated to contain 2 units in 0.2 c.c. The tubes were then reincubated for one hour, placed in the icebox overnight, and read the next morning. The amount of fixation was estimated as 4+ (complete), 3+, 2+, 1+, ± (trace), and 0 (none). The titer was recorded as the highest dilution showing 3+ or 4+ fixation.

A control was always set up with each titration to establish the fact that the antigen was not anticomplementary. This consisted of 0.4 c.c. antigen (1:4), 0.2 c.c. complement (2 units), and 0.4 c.c. sensitized sheep cells.

Results.—The results obtained in the titration of specially prepared rickettsial antigens, when the tests were run at 37° C. with one hour allowed for fixation, are shown in Table I. This table also shows the results obtained on repeated titrations of these suspensions at varying ages.

TABLE I

SPECIALLY PREPARED RICKETTSIAL ANTIGENS TITRATED WITH WATER BATH FIXATION FOR ONE HOUR

SOURCE OF ANTIGEN	STRAIN	DATE TESTED	ANTIGEN DILUTION						USEFUL DILUTION (4 UNITS)
			1:4	1:8	1:16	1:32	1:64	1:128	
National Institute of Health	Endemic 58	2/ 7/44	4	4	4	4	4	2	1:16
		3/20/44	4	4	4	4	4	1	1:16
		6/20/44	4	4	4	3	0	0	1:8
	Endemic 82	3/20/44	4	4	4	4	4	2	1:16
		6/20/44	4	4	4	4	4-	0	1:16
		9/8/44	4	4	4	2	0	0	1:4
		11/28/44	4	4	4-	2	0	0	1:4
	Endemic 84, Lot 3	6/20/44	4	4	4	4	4-	0	1:16
		9/ 8/44	4	4	4	4	0	0	1:8
	Endemic 84, Lot 4	6/20/44	4	4	4	4	4	0	1:16
Sharp & Dohme, Inc.	Endemic	3/20/44	4	4	4	4	4	4	1:32
		11/28/44	4	4	4	4	4	3	1:32
	Epidemic 1	11/28/44	4	2	0	0	0	0	1:1
	2	11/28/44	4	4	2	0	0	0	1:2

From Table I it appears that all of the specially prepared antigens were suitable for use in complement fixation tests and that when freshly made could be used in dilutions of 1:16 or 1:32, that is, using four units in the test, the unit being the smallest amount which gave complete fixation with the standard serum. From Table I it also appears that there was some loss in antigenicity

with the lapse of time though the suspensions were still useful from four to eight months after being initially titrated.

Attention was next directed to a similar investigation of commercially prepared typhus vaccines. For this part of the study, vaccines of various lots representing the finished product of different manufacturers were obtained. These vaccines were all made from the epidemic type virus and the results of their titrations are shown in Table II.

TABLE II

RESULTS OF EPIDEMIC TYPHUS VACCINE TITRATION WITH WATER BATH FIXATION FOR ONE HOUR

SOURCE OF VACCINE	LOT NUMBER	DATE MADE	DATE TESTED	VACCINE DILUTION						USEFUL DILUTION
				1:4	1:8	1:16	1:32	1:64	1:128	
Parke, Davis & Co.	098792 A		9/21/44	4	4-	2	0	0	0	1:2
			11/ 3/44	4	4	1	0	0	0	1:2
	098793 A		9/21/44	4	4	3	tr	0	0	1:4
			11/ 3/44	4	4	3	0	0	0	1:4
	098794 A		9/21/44	4	4-	tr	0	0	0	1:2
			11/ 3/44	4	3	0	0	0	0	1:2
	098795 A		9/21/44	4	4	3	0	0	0	1:4
			11/ 3/44	4	4	2	0	0	0	1:2
Lederle Laboratories, Inc.	285 H 719	4/25/44	9/ 8/44	4	4-	3	0	0	0	1:4
	721	4/26/44	9/ 9/44	4	4	4	1	0	0	1:4
	725	3 /3 and 23/44	9/ 8/44	4	4	3-	0	0	0	1:4
	726	3/ 3 and 22/44	9/ 8/44	4	4-	0	0	0	0	1:2
	739	2/21 and 4/ 5/44	9/ 8/44	4	4-	2	0	0	0	1:2
	43	5/ 1/44	9/ 8/44	4	4-	3	0	0	0	1:4
	45	5/ 2/44	9/ 8/44	4	4-	3	0	0	0	1:4
	50	5/ 8/44	9/ 8/44	4	4-	0	0	0	0	1:2
Eli Lilly and Co.	7084-319035		2/ 7/44	0	0	0	0	0	0	None

The results recorded in Table II indicate that commercially processed typhus vaccines made from the epidemic virus by two manufacturers and representing twelve different lots were all satisfactory for use as antigens in the complement fixation test. Not all the vaccines could be used as antigens in diagnostic tests in the same dilutions, however, as some evidently were suitable only when diluted 1:2, while others could be diluted 1:4. In contrast to these results the product of one company was entirely unsatisfactory as its vaccine was not capable of fixing complement in any dilution in which it was not anticomplementary. Incidentally, it is of interest to note that none of the other vaccines were anticomplementary and that the single unsatisfactory product encountered was that with which Reynolds and Pollard[3] reported obtaining good results. Failure with this product has been reported to us in a personal communication by other investigators.

From Table II it is also seen that the keeping qualities of vaccines to be used as antigens were good. In only one instance was there an apparent loss of antigenicity in the vaccine as manifested by a drop in the dilution which could be made when using it as an antigen.

In contrasting commercial vaccines with specially prepared suspensions of rickettsias as antigens, the advantage lies with the latter, as might be anticipated. This is indicated by the higher antigenic titers obtained and may be accounted for by (1) the greater concentration of the suspensions, (2) the fact that they were prepared from endemic virus, and (3) the use of endemic virus antiserum in carrying out the antigenic titrations.

CONCLUSIONS

1. Specially prepared chick embryo rickettsial suspensions made from epidemic or endemic typhus virus are satisfactory antigens for the complement fixation test.

2. Commercially processed epidemic typhus vaccines representing twelve lots of manufacture were found satisfactory as antigens in complement fixation.

3. No anticomplementary factor was encountered in any of the commercial vaccines shown to be satisfactory as antigens.

4. Typhus vaccine is sufficiently antigenic to be diluted 1:2 or 1:4 in complement fixation.

5. One vaccine was not antigenic in any dilution in which it was not anticomplementary.

6. Typhus vaccines have been found to be satisfactory antigens in complement fixation tests at least for six months after manufacture.

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EFFECT OF PENICILLIN ON EXPERIMENTALLY PRODUCED PLAGUE IN GUINEA PIGS

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INTRODUCTION

SINCE 1910 plague has been present in the northern portion of the Hamakua Coast area of the Island of Hawaii, the largest of the islands comprising the Territory of Hawaii.[1] A number of rats have been found to be infected with the disease each year, and there has been a continued but sporadic occurrence of human cases of bubonic or pneumonic plague. One hundred and eleven human beings have been reported to the health department as having plague during the thirty-five years since the disease first appeared in this district. The maximum number of cases reported for any one year has been twelve. During five yearly intervals no cases were reported.

Plague has occurred in this area more consistently and with greater frequency than in any other area in the United States during recent decades. The mean case rate per 100,000 in the Hamakua area, with a population of approximately 10,000, has been 31.7. In the San Francisco outbreak of 1907 and 1908 the rate was approximately 20 per 100,000 for the two-year period. The rate in the New Orleans outbreak of 1914-1915 was 8.5. Of the patients in the Hamakua area having plague, 110 have died, giving an extraordinary mortality percentage of 99.1. In the San Francisco outbreak seventy-eight of 160 persons contracting the disease died, a mortality of 49 per cent. Of the thirty-one cases in the New Orleans outbreak, ten, or 32 per cent, died. A comparison of plague in these three areas is shown in Table I.

The disease occurs on the Island of Hawaii endemically in rats with an occasional epizootic upswing. Cases in man are sporadic, but the relatively high incidence and high death rate indicate the importance of using this area for studies of plague.

The effect of penicillin on *Pasteurella pestis* naturally was of acute concern, with the hope that this drug might possibly be more effective in treating individuals with plague than any other available medication. The presence of a considerable number of military personnel in the Territory has caused the Armed Forces to be intimately concerned with methods for the suppression of plague here. A protocol for an experiment with penicillin in the treatment of guinea pigs inoculated with plague organisms was prepared by the health department and was submitted to the Army Medical School through the Surgeon of the Central Pacific Base Command. Upon approval of the protocol, sufficient sodium salt of penicillin‡ to conduct the experiment at the Plague Laboratory of the Board of Health at Honokaa, Hawaii, was supplied by the United States

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†President, Board of Health, Territory of Hawaii.

‡Merck & Co., Inc., Rahway, N. J.

TABLE I

COMPARATIVE STATISTICS ON HUMAN PLAGUE IN SAN FRANCISCO, NEW ORLEANS, AND HAMAKUA DISTRICT OF HAWAII

YEAR	CASES	DEATHS	CASE RATE PER 100,000	MORTALITY PERCENTAGE
Hamakua District of Hawaii (1910-1944)				
1910	3	3	30	100
1911	5	5	50	100
1912	7	7	70	100
1913	4	4	40	100
1914	4	4	40	100
1915	4	4	40	100
1916	0	0	0	0
1917	5	0	0	0
1918	0	5	50	100
1919	7	7	70	100
1920	4	4	40	100
1921	4	4	40	100
1922	12	12	120	100
1923	1	1	10	100
1924	2	2	20	100
1925	1	1	10	100
1926	7	7	70	100
1927	7	7	70	100
1928	8	8	80	100
1929	5	4	50	80
1930	0	0	0	0
1931	0	0	0	0
1932	2	2	20	100
1933	2	2	20	100
1934	2	2	20	100
1935	1	1	10	100
1936	0	0	0	0
1937	0	0	0	0
1938	0	0	0	0
1939	1	1	10	100
1940	0	0	0	0
1941	0	0	0	0
1942	0	0	0	0
1943	7	7	70	100
1944	5	5	50	100
Total	111	110	31.7	99.1
San Francisco Outbreak of Plague (Second Outbreak) (May, 1907, to February, 1908)				
1907	158	77	40	49
1908	2	1	0.5	50
Total	160	78	20	49
New Orleans Outbreak of Plague (June, 27, 1914, to Sept. 8, 1915)				
	31	10	8.5	32

Army. The Surgeon General's office stated that at the time penicillin was released for this experiment a similar experiment had been conducted on the mainland on guinea pigs with negative results.

EXPERIMENTS

A pure culture of *Pasteurella pestis* organisms was prepared from a rat infected with plague which was found near the town of Honokaa in the north Hamakua area. The organism was identified by morphology, clinical and pathologic reaction in guinea pigs, and by cultural characteristics. Guinea pigs weighing from 250 to 300 grams were used as experimental animals since guinea pigs have a known marked susceptibility to plague. Two thousand five hundred

organisms, as determined by the McFarland nephelometer and Petroff-Hauser counting chamber, were used in each instance to produce plague in the guinea pigs. Inoculations were made intramuscularly or intraperitoneally into animals previously given penicillin or subsequently treated with the drug. When animals died or were sacrificed, organisms were identified as *Pasteurella pestis* by their morphology in tissue smears and by their biochemical reactions in culture media.

Penicillin Administered Prior to Inoculation with B. pestis (Table II).—One thousand Florey units of penicillin* were injected intramuscularly into eight guinea pigs as follows: 72, 48, 24, 8, 4, 3, 2, and 1 hours prior to sub-

TABLE II

PENICILLIN GIVEN PRIOR TO INOCULATION OF GUINEA PIGS WITH 2,500 B. PESTIS ORGANISMS

GUINEA PIG	DOSAGE AND ROUTE OF PENICILLIN (UNITS/24 HR.)	TIME FIRST ADMINISTERED (HR. PRIOR)	AMOUNT AND ROUTE OF B. PESTIS	TIME OF DEATH AFTER B. PESTIS INOCULATION (HR.)	TOTAL UNITS PENICILLIN ADMINISTERED	DEATH (HR.) AFTER PENICILLIN FIRST ADMINISTERED	ORGANISM RECOVERED
1	1,000 P	72	2,500 S	96	3,000	168	B. pestis
2	1,000 M	72	2,500 P	100	3,000	172	B. pestis
3	1,000 P	48	2,500 S	140	2,000	188	B. pestis
4	1,000 M	48	2,500 P	244	2,000	292	B. pestis
5	1,000 P	24	2,500 S	48	1,000	72	B. pestis
6	1,000 M	24	2,500 P	244	1,000	268	B. pestis
7	1,000 M	8	2,500 S	60	1,000	68	B. pestis
8	1,000 M	4	2,500 S	60	1,000	64	B. pestis
9	1,000 M	3	2,500 S	72	1,000	75	B. pestis
10	1,000 M	2	2,500 S	96	1,000	98	B. pestis
11	1,000 M	1	2,500 S	70	1,000	71	B. pestis

P, Intraperitoneally.
M, Intramuscularly.
S, Subcutaneously.

cutaneous inoculation with *B. pestis* organisms; the dose was repeated at 24-hour intervals for the two animals started on penicillin 72 and 48 hours before receiving the plague organisms. The same dosage of penicillin was injected intraperitoneally into three animals at 24-hour intervals 72, 48, and 24 hours previous to intraperitoneal inoculation with plague organisms. All of these experimental animals died within 244 hours of the time of their inoculation with plague and at autopsy showed plague organisms in smears and bacteriologic cultures made from liver, spleen, and heart blood.

Penicillin Administered at the Same Time as Inoculation With B. pestis (Table III).—Four guinea pigs were given 1,000 units of penicillin, two by intramuscular injection and two by intraperitoneal injection, immediately upon being inoculated with 2,500 *B. pestis* organisms (by the intramuscular route in the case of two animals and by the intraperitoneal route in the other two). The same dosage of penicillin was repeated every 24 hours until death. Four other animals received 300 units of penicillin simultaneously with inoculation with plague organisms and continued to receive 300 units every 3 hours until death. One of these animals was killed after 244 hours. All others died within that period. Cultures and smears at autopsy of these animals showed plague organisms.

*Dosage was based on report by Hamre, Rake, McKee and MacPhillamy on the toxicity of penicillin for guinea pigs.[2]

TABLE III

PENICILLIN STARTED AT TIME OF INOCULATION OF B. PESTIS (2,500) INTO GUINEA PIGS

GUINEA PIG	DOSAGE AND ROUTE OF PENICILLIN	TIME ADMINISTERED	AMOUNT AND ROUTE OF B. PESTIS	TIME OF DEATH AFTER B. PESTIS INOCULATION (HR.)	TOTAL UNITS PENICILLIN ADMINISTERED	DEATH AFTER PENICILLIN FIRST ADMINISTERED (HR.)	ORGANISM RECOVERED
	(UNITS/24 HR.)						
16	1,000 M	Same time as	2,500 P	144	5,000	144	B. pestis
17	1,000 M	Same time as	2,500 S	112	4,000	112	B. pestis
18	1,000 P	Same time as	2,500 P	140	5,000	140	B. pestis
19	1,000 P	Same time as	2,500 S	104	4,000	104	B. pestis
	(UNITS/3 HR.)						
54	300 M	Same time as	2,500 P	144	10,500	144	B. pestis
55	300 M	Same time as	2,500 S	196	14,200	196	B. pestis
56	300 P	Same time as	2,500 P	100	7,500	100	B. pestis
57	300 P	Same time as	2,500 S	Sacrificed after 244	18,400	Sacrificed after 244	B. pestis

P, Intraperitoneally.
M, Intramuscularly.
S, Subcutaneously.

Penicillin Administered Subsequent to Inoculation With B. pestis (Table IV).—Penicillin was injected intramuscularly and intraperitoneally in varying dosages into sixty guinea pigs inoculated either subcutaneously or intraperitoneally with 2,500 *B. pestis* organisms. Treatment was begun 24, 48, 72, 96, or 120 hours after inoculation with plague and continued until death or until 244 hours, when the animals were sacrificed and autopsied. In all of these animals *B. pestis* organisms were seen in large numbers in tissue smears at autopsy. Ten thousand units of penicillin were given every 24 hours to four animals with advanced plague beginning 96 hours after plague inoculation in two animals and 120 hours after plague inoculation in the other two animals, and 100,000 units were given each 24 hours to two animals 96 hours after inoculation with plague organisms. Ten thousand units were given every 3 hours to two animals beginning 120 hours after they had received plague organisms. All of these animals receiving heroic dosages died within 120 hours after penicillin administration and were found to have plague organisms as determined by tissue smears and cultures. Larger dosages were not tried in earlier stages of the disease since Hamre and co-workers[2] found that dosages of from 7,000 to 12,000 units of penicillin per kilogram per day were fatal to guinea pigs.

Controls (Table V).—Ten animals which were inoculated either subcutaneously or intraperitoneally with 2,500 *B. pestis* organisms received no penicillin. One of these died after 48 hours, one after 72 hours, three after 96 hours, two after 112 hours, one after 216 hours, and two were still alive at 316 hours but were sacrificed at that time. *B. pestis* organisms were present in all of these at death, including the two which were sacrificed. Two guinea pigs given 1,000 units of penicillin per day for eleven doses and two given 300 units every 3 hours

TABLE IV

PENICILLIN ADMINISTERED AFTER INOCULATION OF B. PESTIS (2,500) INTO GUINEA PIGS

GUINEA PIG	DOSAGE AND ROUTE OF PENICILLIN	TIME ADMINISTERED (HR. AFTER)	AMOUNT AND ROUTE OF B. PESTIS	TIME OF DEATH AFTER B. PESTIS INOCULATION (HR.)	TOTAL UNITS PENICILLIN ADMINISTERED	DEATH AFTER PENICILLIN FIRST ADMINISTERED (HR.)	ORGANISM RECOVERED
	(UNITS/24 HR.)						
20	1,000 M	24	2,500 P	86	2,000	62	B. pestis
21	1,000 M	24	2,500 S	72	1,000	48	B. pestis
22	1,000 P	24	2,500 P	86	2,000	62	B. pestis
23	1,000 P	24	2,500 S	172	5,000	148	B. pestis
24	1,000 M	24	2,500 P	124	3,000	100	B. pestis
25	1,000 M	48	2,500 S	96	1,000	48	B. pestis
26	1,000 P	48	2,500 P	124	3,000	72	B. pestis
27	1,000 P	48	2,500 S	196	6,000	148	B. pestis
28	1,000 M	48	2,500 P	176	5,000	128	B. pestis
29	1,000 M	48	2,500 S	144	3,000	96	B. pestis
30	1,000 P	72	2,500 P	200	5,000	128	B. pestis
31	1,000 P	72	2,500 S	152	3,000	80	B. pestis
32	1,000 M	72	2,500 P	121	2,000	49	B. pestis
33	1,000 M	72	2,500 S	121	2,000	49	B. pestis
34	1,000 P	96	2,500 P	98	1,000	2	B. pestis
35	1,000 P	96	2,500 S	224	5,000	128	B. pestis
36	1,000 M	96	2,500 P	230	5,000	134	B. pestis
37	1,000 M	96	2,500 S	196	4,000	100	B. pestis
38	1,000 P	96	2,500 P	200	4,000	104	B. pestis
39	1,000 P	120	2,500 S	Sacrificed after 244	4,000	Sacrificed after 124	B. pestis
41	1,000 M	96	2,500 S	Sacrificed after 244	5,000	Sacrificed after 148	B. pestis
42	10,000 P	96	2,500 P	148	20,000	52	B. pestis
43	1,000 M	96	2,500 S	200	4,000	104	B. pestis
44	10,000 P	96	2,500 P	196	40,000	100	B. pestis
45	100,000 P	96	2,500 S	172	300,000	76	B. pestis
46	100,000 P	96	2,500 P	172	300,000	76	B. pestis
47	10,000 M	120	2,500 S	240	50,000	120	B. pestis
48	10,000 P	120	2,500 P	240	50,000	120	B. pestis
50	1,000 M	120	2,500 P	216	4,000	96	B. pestis
51	1,000 P	120	2,500 S	172	3,000	50	B. pestis
	(UNITS/3 HR.)						
58	300 M	24	2,500 P	96	5,400	72	B. pestis
59	300 M	24	2,500 S	Sacrificed after 244	16,600	Sacrificed after 220	B. pestis
60	300 P	24	2,500 P	Sacrificed after 244	16,600	Sacrificed after 220	B. pestis
61	300 P	24	2,500 S	220	14,800	196	B. pestis
62	300 M	48	2,500 P	172	9,300	124	B. pestis
63	300 M	48	2,500 S	112	4,800	64	B. pestis
64	300 P	48	2,500 P	72	1,000	24	B. pestis
65	300 P	48	2,500 S	148	9,000	100	B. pestis
66	300 M	72	2,500 P	148	7,200	72	B. pestis
67	300 M	72	2,500 S	172	9,000	100	B. pestis
68	300 P	72	2,500 P	130	6,000	58	B. pestis
69	300 P	72	2,500 S	114	5,100	42	B. pestis
70	300 M	96	2,500 P	148	3,600	52	B. pestis
71	300 M	96	2,500 S	116	3,000	20	B. pestis
72	300 P	96	2,500 P	196	9,000	100	B. pestis
73	300 P	96	2,500 S	196	9,000	100	B. pestis
74	300 M	120	2,500 P	Sacrificed after 244	12,000	Sacrificed after 124	B. pestis
75	300 M	120	2,500 S	172	5,700	52	B. pestis
76	300 P	120	2,500 P	196	7,500	76	B. pestis

P, Intraperitoneally.
M, Intramuscularly.
S, Subcutaneously.

TABLE IV—CONT'D

GUINEA PIG	DOSAGE AND ROUTE OF PENICILLIN	TIME ADMINISTERED (HR. AFTER)	AMOUNT AND ROUTE OF B. PESTIS	TIME OF DEATH AFTER B. PESTIS INOCULATION (HR.)	TOTAL UNITS PENICILLIN ADMINISTERED	DEATH AFTER PENICILLIN FIRST ADMINISTERED (HR.)	ORGANISM RECOVERED
	(UNITS/3 HR.)						
77	300 P	120	2,500 S	192	6,900	72	B. pestis
78	5,000 M	120	2,500 P	196	7,500	76	B. pestis
79	5,000 P	120	2,500 S	Sacrificed after 244	205,000	Sacrificed after 124	B. pestis
80	10,000 M	120	2,500 P	148	90,000	28	B. pestis
81	10,000 P	120	2,500 S	172	170,000	52	B. pestis
82	1,000 M	120	2,500 P	172	17,000	52	B. pestis
83	1,000 P	120	2,500 S	196	25,000	76	B. pestis
85	5,000 M	144	2,500 S	196	85,000	52	B. pestis
86	5,000 P	144	2,500 P	196	85,000	52	B. pestis
87	5,000 M	144	2,500 S	196	85,000	52	B. pestis
88	1,000 P	144	2,500 P	Sacrificed after 244	33,000	Sacrificed after 100	B. pestis

P, Intraperitoneally.
M, Intramuscularly.
S, Subcutaneously.

TABLE V

CONTROL GUINEA PIGS INOCULATED WITH B. PESTIS (2,500)

GUINEA PIG	DOSAGE AND ROUTE OF PENICILLIN	TIME ADMINISTERED	AMOUNT AND ROUTE OF B. PESTIS	TIME OF DEATH AFTER B. PESTIS INOCULATION (HR.)	TOTAL UNITS PENICILLIN ADMINISTERED	DEATH AFTER PENICILLIN FIRST ADMINISTERED (HR.)	ORGANISM RECOVERED
	(UNITS DAILY)						
12	1,000 M	72 hr. before	–	Sacrificed after 316	11,000	Sacrificed after 316	None
15	1,000 P	72 hr. before	–	Sacrificed after 316	11,000	Sacrificed after 316	None
	UNITS/3 HR.						
52	300 M	Same time as	–	Sacrificed after 244	19,200	Sacrificed after 244	None
53	300 P	Same time as	–	Sacrificed after 244	19,200	Sacrificed after 244	None
40	–	–	2,500 P	72	–	–	B. pestis
49	–	–	2,500 S	112	–	–	B. pestis
84	–	–	2,500 P	48	–	–	B. pestis
89	–	–	2,500 S	216	–	–	B. pestis
90	–	–	2,500 P	96	–	–	B. pestis
91	–	–	2,500 S	112	–	–	B. pestis
92	–	–	2,500 P	96	–	–	B. pestis
93	–	–	2,500 S	96	–	–	B. pestis
13	–	–	2,500 P	Alive; sacrificed after 316	–	–	B. pestis
14	–	–	2,500 S	Alive; sacrificed after 316	–	–	B. pestis
98 Normal control	–	–	–	Alive		–	–

P, Intraperitoneally.
M, Intramuscularly.
S, Subcutaneously.

until a total of 19,200 units had been given showed no symptoms of illness and no gross or microscopic pathology at autopsy after being sacrificed. Although the widespread variation in the time of death after plague inoculation of the control animals and of the animals receiving penicillin does not permit any accurate statistical comparison, it can be said that there was no apparent prolongation of life caused by penicillin administration.

TABLE VI

IN VITRO EFFECT OF PENICILLIN ON B. PESTIS AT 29° C. (2,500,000 B. PESTIS ORGANISMS IN 1 C.C. OF PHYSIOLOGIC SALINE SOLUTION)

TOTAL UNITS OF PENICILLIN (IN 10 C.C. DUNHAM'S PEPTONE)	ORGANISM RECOVERED AFTER 6 DAYS
10	B. pestis
100	B. pestis
1,000	B. pestis
10,000	B. pestis
20,000	B. pestis
30,000	B. pestis
40,000	B. pestis
50,000	B. pestis
60,000	B. pestis
70,000	B. pestis
80,000	B. pestis
90,000	B. pestis
100,000	None
200,000	None
300,000	None
400,000	None
500,000	None

In Vitro (Table VI).—Sterile test tubes containing 10 c.c. each of Dunham's peptone in which varying concentrations of penicillin had been dissolved were each seeded with 1 c.c. of a saline suspension of a 24-hour veal-infusion agar growth of *Pasteurella pestis*. The *B. pestis* suspension contained approximately 2,500,000 organisms per cubic centimeter. These tubes were incubated at 29° C. for six days and transplants made to test for sterility. Concentrations of penicillin of 100,000 units or greater were required to kill the inoculum of 2,500,000 *B. pestis* organisms.

CONCLUSIONS

1. Plague is a serious public health problem in the north Hamakua area of the Island of Hawaii.

2. In this experiment penicillin appeared to be of no benefit in combating bubonic plague in guinea pigs.

3. In vitro, a concentration of 100,000 units of penicillin in Dunham's peptone solution was required to kill an inoculum of 2,500,000 *B. pestis* organisms when incubated at 29° C. for six days.

The authors wish to express their appreciation to Sgt. George T. O'Dea and Pfc. John Wilson for their assistance in conducting the in vivo experiments.

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CONGENITAL HEMOLYTIC ANEMIA IN A NEGRO

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CONGENITAL hemolytic icterus has been reported only once in the Negro race.[1] Because the condition apparently is rare in this race, it seems worth while to report the occurrence of such a case which has come under our observation.

CASE REPORT

A. F., a 14-year-old, dark-skinned Negro girl, was admitted to St. Philips' Hospital April 17, 1944, because of anemia and a low-grade fever of undetermined origin. She had been well until eleven weeks previous to admission. At that time she had developed a sore throat followed by a painful right wrist with no further joint symptoms. At the time of the first examination, her physician found the hemoglobin to be 6.5 Gm. per 100 c.c., and, as she continued to run a low-grade fever, she was referred to the local hospital. There the hemoglobin was recorded as 9.0 Gm., and it was noted that there was enlargement of the heart and spleen. She was given one transfusion and intramuscular liver and bismuth injections, the latter because of the finding of a positive Wassermann. She left the hospital apparently unimproved, and in the interim prior to her admission here, two x-ray pictures of the chest, made because of slight loss of weight, were negative.

Past history was essentially negative.

Family history: The mother died when the patient was 9 days old. No information was available as to the cause of death. The father and one brother are living and in good health. There were no other children.

Physical examination revealed a well-developed, well-nourished Negro girl, with the usual dark color seen in the average Negro in this region, lying in bed in no acute distress; temperature 101° F.; pulse, 134; respirations, 34. There was a slight icteric tint to the sclerae. The heart was moderately enlarged to the left. Roentgenogram of the chest showed a mitral configuration of the heart. There was a loud blowing systolic murmur over the entire precordium, best heard over the pulmonic area. No thrills were noted. Blood pressure was 144/80. The cardiac consultant believed the cardiac findings to be those of chronic anemia. The spleen was markedly enlarged and presented a firm sharp edge. There was slight edema of both ankles. The cervical, axillary, and inguinal nodes were moderately enlarged, discrete, and nontender. All other findings were essentially normal.

Laboratory examination: Electrocardiogram showed sinus tachycardia, a rate of 125 to 136 per minute; no right axis deviation. Urinalysis was normal. Blood examination: red blood cells, 2,520,000 per cubic millimeter; hemoglobin, 6.2 Gm. per 100 c.c.; white blood cells, 15,600, with 82 per cent polymorphonuclear neutrophiles, 1 per cent eosinophiles, 1 per cent basophiles, 14 per cent lymphocytes, 2 per cent monocytes; reticulocytes, 17 per cent; icteric index, 16 units. Fragility test using the method of Sanford revealed hemolysis beginning at 0.75 and complete at 0.42; controls, 0.44 to 0.32. The red blood cells did not show the sickling phenomenon. The Wassermann test was negative.

Course: In view of these findings, congenital hemolytic jaundice was thought to be the most plausible diagnosis. The blood film showed typical microspherocytosis with a cell diameter of 7 microns. The fragility test when repeated showed hemolysis starting at 0.70 and completed at 0.42. Biopsy of an axillary lymph node showed moderate hyperplasia with scattered reticulum cells containing blood pigment. With rest in bed, the anemia improved and April 29, the date of splenectomy, the red blood cell count was 2,900,000 per cubic millimeter; the hemoglobin, 8.7 Gm.; and the icterus index, 16 units.

At operation a spleen weighing 465 grams was removed. There was some accessory splenic tissue at the hilus of the organ. The capsule was smooth and glistening and was a

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deep bluish-purple color mottled with several irregular reddish-purple areas. On section the follicles appeared quite prominent, but there appeared to be only a slight increase of fibrous trabeculation. The organ was somewhat firmer than usual. Microscopically, the spleen showed prominent, irregular sinuses and marked congestion of pulp with many cells containing blood pigment. It was thought that the appearance was consistent with the spleen of congenital hemolytic icterus.

By May 14 the patient was greatly improved, and laboratory examination revealed the following: red blood cells, 3,580,000 per cubic millimeter; hemoglobin 10.6 Gm.; white blood cells, 11,150, and a normal differential. Fragility began at 0.60 and was complete at 0.33. This was repeated May 17 when hemolysis began at 0.54 and was complete at 0.39. Microspherocytosis was present and there appeared to be no increased regeneration. The patient was discharged May 20 in excellent condition.

She returned for re-examination Aug. 10, 1944, and appeared to be in perfect health. The cardio-thoracic ratio was found to be reduced to 46 per cent as compared to 55 per cent when the patient was in the hospital before splenectomy. The systolic murmur, believed to be functional, was still present. Laboratory examination showed the following: red blood cells, 4,490,000; hemoglobin, 15.3 Gm.; white blood cells 5,600; and an icterus index of 8 units. Hemolysis began at 0.54 and was complete at 0.30. The blood films showed microspherocytes.

FAMILY STUDY

The family was well established in a rural community of Virginia where its members had resided for some years. Questioning of white persons in the community did not shed any light on the possibility of the presence of white blood in the family background. A fourth-year medical student from the community carried out this survey, securing blood films on the various members of the family. These were studied by Dr. Russell Haden and the results are reported in Table I. Three other members of the family who visited the hospital were also studied, although mean cell diameters were not obtained. In none was the spleen palpable. Studies on these are recorded in Table II.

TABLE I

RELATIONSHIP	COLOR	MICRO-SPHEROCYTOSIS	MEAN CELL DIAMETER
1. Patient	Dark	+	7.0
2. Aunt on father's side	Tan	-	8.0
3. Grandfather on father's side	Tan	-	7.9
4. Grandmother on father's side	Tan	-	7.7
5. Uncle on mother's side	Tan	-	8.0
6. Uncle on mother's side	Tan	+	6.6
7. Grandmother on mother's side	Dark tan	+	7.2
8. Uncle on father's side	Quite light	-	8.0
9. Aunt on father's side	Typical Negro	-	8.0

Blood films obtained from the father and uncle showed small cells, many well filled with hemoglobin and highly suggestive of microspherocytosis. The increased reticulocytes in these two, as shown in Table II, in the light of the high hemoglobin is interesting as is the increased icterus index. Films from the brother showed no microspherocytes, although the cells appeared small for an 18-year-old boy.

TABLE II

	HEMOGLOBIN (PER CENT)	RETICULOCYTES (PER CENT)	ICTERIC INDEX (UNITS)	FRAGILITY
Father	110	3	8	0.42 to 0.30
Uncle on father's side	94	3.2	10.5	0.42 to 0.30
Brother	102	1.2	4.5	0.48 to 0.33

DISCUSSION

Many textbooks describe congenital hemolytic icterus as occurring "in all races and climates." However, one of us (J. H. S.), in a relatively extensive experience with the blood of Negroes, had never encountered the disease in the race. After ten years' experience as hematologist associated with the large Negro hospital and clinics of the Medical College of Virginia, he had begun to feel that congenital hemolytic icterus did not occur in the Negro, sickle-cell anemia possibly being its counterpart.

Wintrobe[1] states that he has "encountered one instance (of congenital hemolytic icterus) in a Negress of undoubtedly mixed blood." Various reviews[2-6] do not record the disease in the Negro race. Haden[7] and Kracke,[8] both interested in congenital hemolytic icterus, have not observed the disease in a Negro.

The significantly altered erythrocyte diameters in the family group are in the uncle on the mother's side, with a mean cell diameter of 6.6, and the grandfather on the mother's side. The uncle was described by the investigator as "tan," the grandfather as "dark tan."

A confusing finding is the definite microspherocytic tendency with increased reticulocytes and icteric index on the father's side. It would be assuming too much to suggest the presence of the trait on both sides of the family. The father could recall no history of jaundice in the family.

It is possible that congenital hemolytic anemia does occur in full-blooded Negroes, although it has not been recorded previously. Infectious mononucleosis, for instance, only recently has been observed in the race. It may well be that less adequate hematologic studies have contributed to this oversight. On the other hand, the interbreeding of the trait through miscegenation in the family background cannot be excluded. The disease in the Negro must be rare, and this rarily is to be expected if the trait is found in the Negro only after inbreeding. Congenital hemolytic anemia is a comparatively uncommon disease in the white race. The probability of transmission through miscegenation must be infinitesimal.

SUMMARY

The first case of congenital hemolytic anemia to be reported in detail in the Negro race is recorded. Apparently the disease, though rare, does occur among Negroes. The probability of transmission of the trait through miscegenation is discussed.

Tht authors wish to acknowledge the kindness of Dr. Russell L. Haden, for measuring the red cell diameters, and of Mr. E. G. Field, for conducting the family survey.

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THE LIFE DURATION OF THE RED BLOOD CELL OF THE MACACUS RHESUS MONKEY

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MANY efforts have been made to establish the life duration of the erythrocyte in the circulating blood. Such diverse mammalian forms as the rat, rabbit, dog, monkey, and man have been studied and many different approaches to the problem have been used. It is not the purpose of this paper to cover the literature on the subject more than to indicate the chief avenues of attack.

Ashby[1] employed differential agglutination of transfused blood cells as an index of their persistence and concluded that the erythrocyte of man possessed a life duration of thirty days or more. Later, Isaacs[2] showed that the agglutination response could not be relied upon to identify transfused red cells beyond the second to the fourth day following transfusion. Eaton and Damren[3] utilized the young erythrocyte (or reticulocyte) as an indicator of red cell replacement following hemorrhage. They showed that in the rabbit following hemorrhage new groups of reticulocytes invade the circulation rhythmically at intervals of from eight to nine days. Such intervals were interpreted as representing the life duration of the red blood cell in this animal. Escobar and Baldwin[4] studied the effects of altered oxygen tension on the blood of rats. The time required for restoring the erythrocyte count to normal following lowered oxygen tension was interpreted as representing the life duration of the circulating red blood cell. This they found to be from eleven to thirteen days. Hawkins and Whipple[5] employed bile pigment output as an indicator of erythrocytic disintegration. These workers found the interval between a sudden blood depletion in a dog and a subsequent sudden rise in bile pigment output to average 124 days. This time interval, they believe, represents the days between the influx of an erythrocyte population induced by sudden blood cell depletion and the subsequent time of disintegration of this cell group and hence represents the life of the red blood cell of the dog. Harne and associates[6] using the approach of Eaton and Damren, studied the blood of the albino rat. It was found that the life span of the red blood cell was between eight and nine days. This life span agrees with that of the rabbit.

In the present study monkeys were subjected to acute hemorrhage and the changes in the blood picture which follow were observed daily. Not all these observations will be discussed here. In this paper we shall consider the experimental procedure and those results which relate specifically to the life span of the red cell.

It has been established in all animal forms thus far studied that when large numbers of red cells are removed from an animal's circulation by hemorrhage, the cells which are thus lost will be promptly replaced by a large number of

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reticulocytes. When supravitally stained, these reticulocytes can be identified and counted. Thus by using the reticulocyte count as an indicator, it is possible to evaluate the degree of red cell replacement.

It is obvious that when a population of circulating red blood cells is abruptly removed, as by hemorrhage, and a group of young cells promptly replaces those lost, there will exist in the blood stream a group of red cells all of which are approximately the same age. If all red cells have about the same span of life, a block of such young cells should mature together and go on through life as a functioning unit, finally to become senile and disappear en masse. This physiologic depletion of a cell population simulates in effect a depletion by hemorrhage and thus spontaneously initiates a replacement reticulocyte shower. This spontaneous replacement of cells can again be detected and evaluated as before by the reticulocyte count. Such a sequence of reactions has been shown to occur in all animal forms thus far tested.[3, 6]

The time lapse between the appearance of the first replacement block of young red cells following hemorrhage and that of the second (spontaneous) block should express the life tenure of the red blood cell in the animal studied. This paper presents the evidence of such a sequence as it occurs in the monkey.

ANIMAL MATERIAL

Ten young macacus rhesus monkeys were used, four males and six females. The ages at the beginning of the experiment (September, 1941) were estimated to be between 6 and 7 years (Table I). They were kept in separate cages except when permitted to exercise together. Each animal was fitted with a stout collar and a chain by which it could be controlled. For a period of several months preceding experimentation all were schooled to leave the cage and climb upon a table located near by. Here they were covered with a net which was secured

TABLE I

SHOWING AGE, SEX, WEIGHT, AND CONDITION OF ANIMAL MATERIAL WITH NOTES ON TRAINING AND INDIVIDUALITY

ANIMAL NO.	SEX	ESTIMATED AGE (YR.)	WEIGHT (KG.)	PHYSICAL CONDITION	REACTION TO TRAINING	NOTATIONS ON INDIVIDUALITY
1	F	7	4.6	Good	Poor	Never became tame; would nervously assist operator with restraining net; excellent subject
2	F	7	3.6	Good	Fair	Ugly, mean, and thievish
3	F	7	4.	Good	Excellent	Good subject; bossy; amorous
4	F	7	4.	Good	Excellent	Good subject; shakes hands timidly; shy
5	M	7	4.	Good	Excellent	Good subject; would be boss of colony
6	M	7	4.4	Good	Excellent	Good subject; vain; thievish; very playful
7	M	6	4.4	Good	Good	Good subject; big bluffers; playful
8	M	6	4.	Good	Poor	Does only what he has to
9	F	6	3.6	Good	Fair	Responsive; devilish
10	F	6	3.4	Good	Fair	Responsive but does things her own way

to the table top by means of a system of sliding blocks. Under the net the animals were petted and offered favorite articles of food.

Eventually they would accept the food and sit quietly while being handled. Each responded in a different way to the total treatment, but all learned to climb upon the table and wait to be covered with the net. One animal (No. 1) actually learned to assist the technician with the preparations (Table I). At no time during the experiment did we notice any evidence of resentment or objection to the tests.

The net used was an ordinary pickerel net 18 inches deep, mounted upon a square frame made from ⅜ inch metal rod bent into a 14-inch square. The ends of the rod were bent out and brazed together to form a handle.

Taking the Blood.—Blood for the various tests was obtained by three different methods. Small amounts of blood for the hemoglobin estimation and reticulocyte, differential, red and white cell counts were taken from the ear

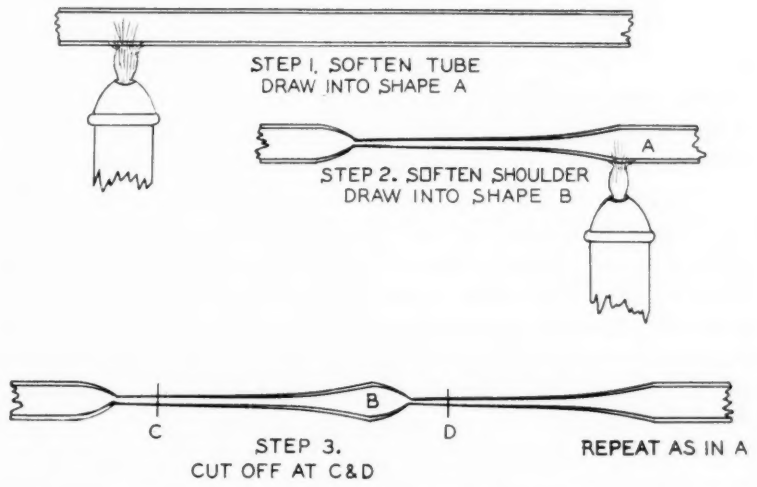

Fig. 1.—Showing steps in the fabrication of the blood-collecting and staining pipette.

by quickly snipping the free auricular border with a pair of small sharp scissors. Only the reticulocyte preparations so obtained are further considered in this paper. Larger amounts of blood, 1 to 5 c.c. for hematocrit determinations, were taken from the superficial veins of the legs, and the largest amounts, 5 to 50 c.c., the so-called hemorrhage, used to induce reticulocytosis, were taken from the femoral vein after the latter had been exposed under light anesthesia.*

Preparation of Reticulocyte Films.—The best preparations of reticulocytes in monkey blood were obtained by the pipette method. This requires a blood pipette made for the purpose and a proper staining solution.

Pipette.—The pipette used (Fig. 1, *B*) is spindle shaped and about 12 cm. long. The caliber at the tip is about 1.5 mm. and at the expanded central portion, about 6 to 8 mm. The distal end is not important except that it must be open. The pipette can be drawn into shape from a piece of ordinary glass tubing 15 mm. in diameter. In fabricating it, the end of the tube is first drawn

*Anesthesia was induced by the intravenous injection of a fresh aqueous solution of 4 per cent Nembutal—3/4 c.c. per kilogram body weight.

out to the length and diameter required to form the tip of the pipette. The flame of the blast lamp is then played upon the shoulder of the drawn out portion (Fig. 1, *A*) to soften it just before it is pulled into shape *B*, Fig. 1. This method gives a good taper to the spindle and a sufficiently large bulb to insure proper functioning.

The Staining Solution.—The stain is prepared as follows:

Brilliant cresyl blue	1 per cent
Potassium oxalate	.5 per cent
Merthiolate (1-1,000)	.025 per cent
Sodium chloride	.8 per cent

Dissolve the brilliant cresyl blue in distilled water at room temperature by shaking for fifteen minutes in a shaker. Let stand overnight. Shake again for fifteen minutes and filter through a medium textured paper which is rapid and fairly retentive. Add the oxalate, merthiolate, and sodium chloride. Make up to volume with distilled water and the stain is ready for use. The solution keeps very well but must be stored in a tightly stoppered bottle.

Use of the Pipette.—Hold the pipette in a *vertical* position; touch its tip to the surface of the staining solution. Capillary attraction will cause the stain to rise in the pipette to a distance of from 1.5 to 2 cm., depending upon the caliber of the capillary. The stain will remain in position until used unless it is handled roughly or touched against an absorbent. The stain should not be kept in the pipette too long or evaporation will change its ion concentration and so affect its isotonicity with blood.

Hold the pipette in a horizontal position and touch the tip, filled to the end with stain, to the surface of the freshly formed drop of blood. Blood at once enters the pipette. The amount of blood so admitted is not too important, but it is well to limit the quantity to an amount equal to the volume of stain used. *Mix* the stain and blood promptly by first holding the tip of the pipette up until the mixture flows down almost to the bulb and then quickly turning the tip down. Do this several times, or until mixing is complete. Do not hold the pipette tip up too long or the blood will flow into the opposite end of the pipette, air-locking the contents. This does not harm the preparation but makes it very difficult to agitate and remove the mixture. *Smears* should be made from the pipette after about five minutes. First agitate the contents as described, and then immediately place a small drop of the mixture upon the surface of a *perfectly clean* slide and spread it in the usual manner, using the end of another clean slide as a spreader. Allow the films to dry in the air; mount in balsam if permanent preparations are desired. *Examination* of the films may be made immediately without counterstaining, but some prefer to counterstain with Wright's blood stain. Whether counterstained or not, these preparations hold up very well.

Another type of preparation is now being used extensively and can be made from the blood-stain mixture in the pipette as follows: Place a medium sized drop of the blood-stain mixture in the center of a *clean slide* and cover it with a *clean* cover slip. Seal the edges of the cover slip with paraffin. Allow to stand for a few minutes to permit the cells to settle and examine as usual.

This so-called "wet-preparation" is by far the best method for studying the cells and their inclusions. The preparations so made, however, are *not* permanent.

Counting the Reticulocytes.—The reticulocyte count is accomplished by first exploring the smear under low power for an area in which the cells are evenly distributed and moderately separated. The area thus located is then studied under oil immersion and usually found to contain about 100 red cells per field. Starting from a given point within this area, and counting adjacent fields, 1,000 red cells are counted. The reticulocytes are recorded separately for each block of 100 red cells. This block system of recording is employed to give data on the distribution of the reticulocytes within the area counted. Upon completion of the count the reticulocytes are summed up and expressed in per cent or, better still, as reticulocytes per 1000 red cells.

Reticulocyte Level in the Monkey Under Control Conditions.—Tests were begun as soon as the animals became accustomed to the training routine. Each animal underwent standardization of his own reticulocyte output by being subjected to daily reticulocyte counts. Experience showed that from ten to twenty days were sufficient to get the pattern of reticulocyte production (Table II).

Two animals (Nos. 2 and 6, Fig 2) were kept as controls throughout the course of the experiments. Both were ultimately subjected to acute hemorrhage and reacted as normal animals to this procedure. The terminal record of Animal No. 2 appears as No. 2, Series 2, Fig. 4. The terminal record of Animal No. 6 does not appear, as the experiment was terminated after the initial reaction. In this way, all animals of the colony contributed to the results reported here. The combined control and experimental periods of Animals 2 and 6 afforded continuous daily records of the reticulocyte counts for 287 and 220 days, respectively.

The average control level of reticulocytes for the colony (ten animals) was 10.9 reticulocytes per 1,000 red cells. Coincidently the average reticulocyte level of the male control animals (No. 6), whose control period extended over a period of 174 days, was also 10.9.

The average control count for the males (four animals) was 10.3 and for the females (six animals), 11.3 per 1,000 red cells.

The highest average level in a single animal was 16.7 per 1,000 over a twenty-day period, found in a female (No. 1, Series 2, Table II) also (No. 1, Series 2, Fig. 4). The lowest record, 8.5 per 1,000 over a 70-day period, was shared by two males (Nos. 7 and 8, Table II, and Nos. 7 and 8, Fig. 3). The initial high level of Animal 1 was accepted as a control value because of its constancy even though the value seemed high. Subsequent analyses of these data show that this was not an error.

It is seen (Fig. 2) that the average value of 10 reticulocytes per 1,000 is about correct for the monkey when determinations are carried out over long periods, as this line cuts the long control curves of Animals Nos. 2 and 6 through the center. There are long periods, however, when the curves rise above or fall below this level. We believe, therefore, that the variation in the control levels of the experimental animals which showed variations can be ascribed to the varying activity of cell production at the time the control tests were made

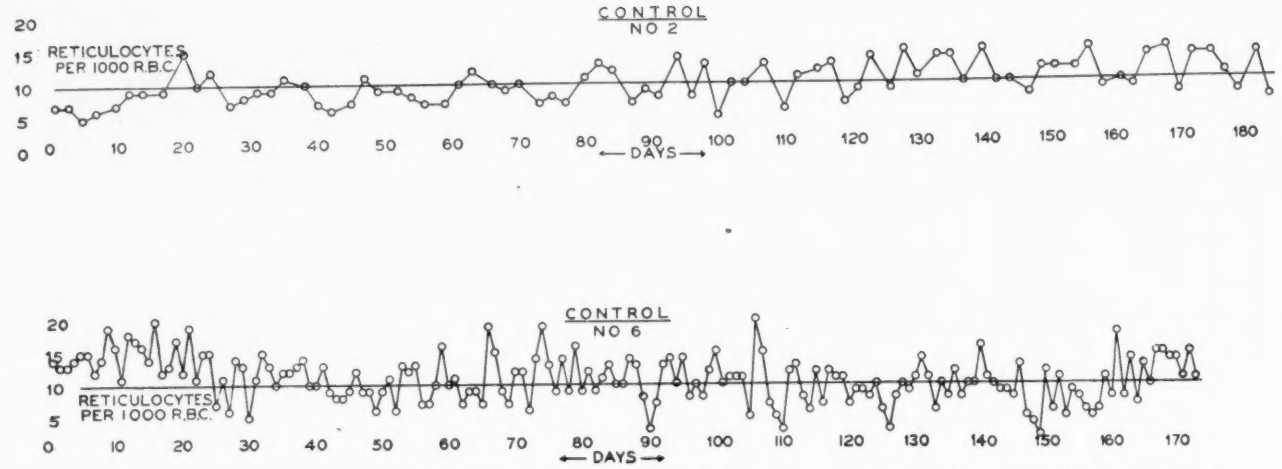

Fig. 2.—Showing control reticulocyte counts in Animals Nos. 2 and 6 over periods of 185 and 174 days, respectively.

TABLE II

SHOWING (1) LENGTH OF THE CONTROL PERIOD, (2) TYPE AND AMOUNT OF HEMORRHAGE, (3) CONTROL RETICULOCYTE LEVEL AS FOUND BY DAILY COUNTS, AND (4) LENGTH OF LIFE OF RED BLOOD CELL

ANIMAL NO. AND EXPERIMENT NO.	NAME AND SEX	CONTROL PERIOD (DAYS)	TYPE OF HEMORRHAGE AND AMOUNT	AVERAGE CONTROL LEVEL RETICULOCYTES (PER 1,000 R.B.C.)	LENGTH OF LIFE RED BLOOD CELL (DAYS)
1	Jiggs F	20	Acute 40 c.c.	8.8	105
1, Series 2	Jiggs F	20	Fractional 9-9-9-9 c.c.	16.7	103
2	Susie F	185	Control	10.5	Control
2, Series	Susie F	10	Fractional 10-10-10-10 c.c.	16.1	112
3	Oscar F	20	Acute 41 c.c.	9.2	94
4	Windy F	20	Fractional 12.5-19-15-16 c.c.	11.2	101
5	Spike M	20	Fractional 23-23-23-23 c.c.	14.3	102
5, Series	Spike M	20	Acute 43 c.c.	10.	103
6	Pete M	174	Control	10.9	Control
7	Bufo M	70	Acute 40 c.c.	8.5	111 Estimated
8	Lightning M	70	Acute 43 c.c.	8.5	117
9	Flo F	81	Acute 27 c.c.	11.2	
10	Tess F	81	Acute 34 c.c.	12.2	

and is to be expected when *short* control periods are used. Animal No. 1 was a good example. This animal gave widely different control values at periods separated by intervals of six months (16.7 and 8.8, respectively), using twenty days in each case as the control period.

The Effect of Hemorrhage Upon the Hemopoietic System.—Following the establishment of individual control reticulocyte values, eight of the ten animals were bled. The amount and type of hemorrhage so induced are shown in Table II and in Figs. 3 and 4. Subsequent to bleeding, daily reticulocyte counts were continued for a period of approximately 130 days and the data so obtained plotted. The resulting curve resolves itself into three parts. The first or initial phase extends over a period of from twenty to thirty days. During this phase the reticulocytes rapidly increase in number and then subside to control level or lower. In the second or interval period the reticulocyte count remains at approximately control level. This extends to about the eightieth day, following which a recrudescence of reticulocytes produces a second elevation or spontaneous phase. The latter and its return to control level constitute the terminal phase of the curve. The first elevation is interpreted as a mass influx of red cells from the bone marrow to replace the loss occasioned by bleeding. The second period is regarded as a stabilized phase following the

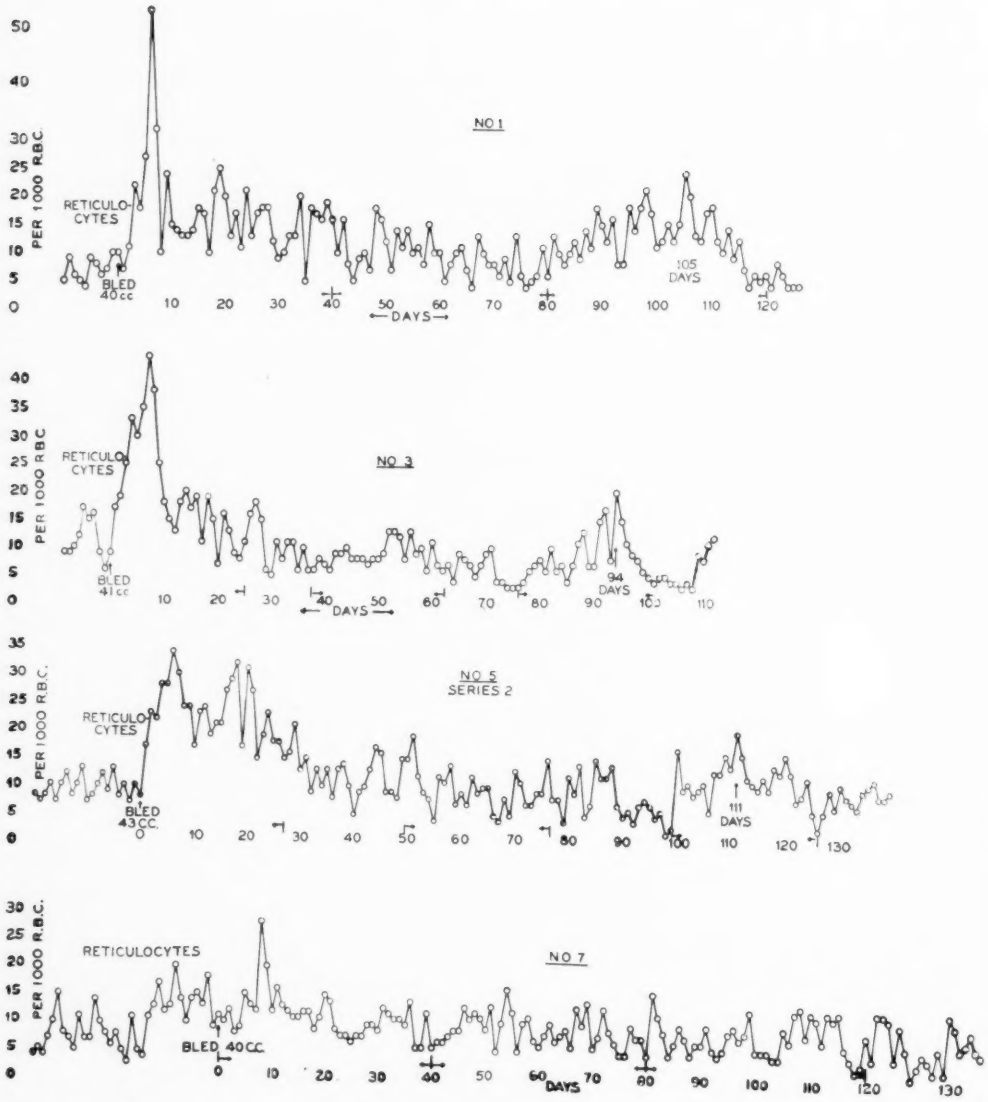

Fig. 3.—Showing reticulocyte counts following the withdrawal of blood equivalent to 1 per cent of body weight at one operation.

restoration of the blood population to normal. The third period represents a spontaneous reticulocytosis, the function of which is to replace the group of cells released during the first period following the hemorrhage and which are now disappearing more or less in mass from the circulation.

This three-phase type of curve has been obtained in eight of eleven experiments (Table III). In seven experiments, the animals were deprived of blood equivalent to 1 per cent of their body weight by a single operation (Fig. 3). In four experiments the animals were bled fractionally; approximately .5 per cent of the body weight in blood was removed at intervals of seventy-two hours (Fig. 4 and Table II). All animals (four) bled fractionally gave the three-

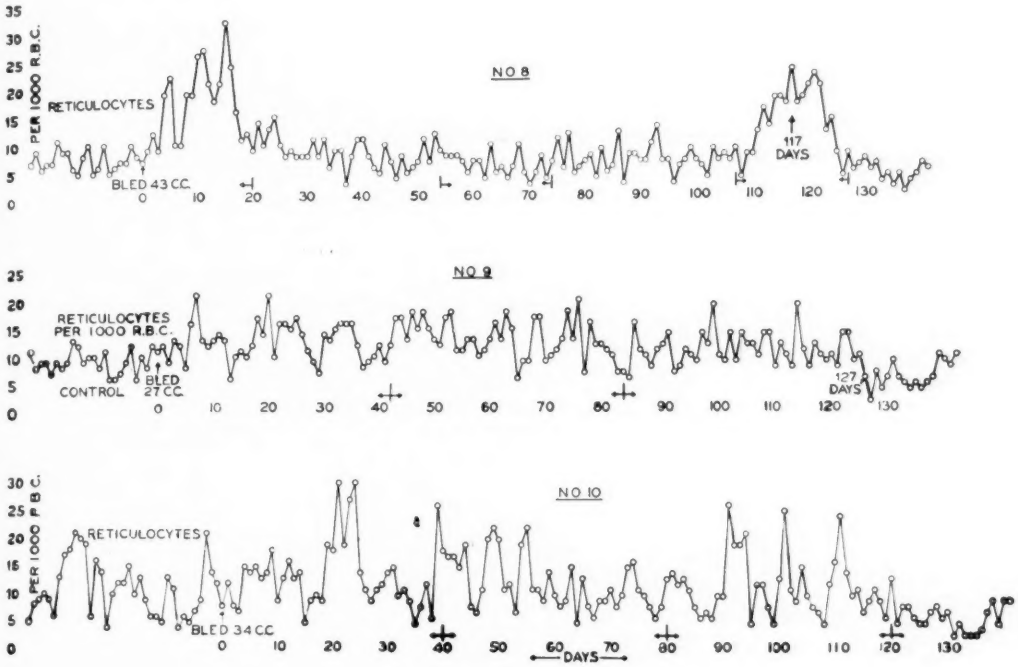

Fig. 3.—(Cont'd.)

phase type of curve described. It should be pointed out, however, that the interval period in each of these experiments shows qualitative deviations from the classical picture and are not as smooth as the more typical curves in Fig. 3, which were obtained by single acute withdrawals of blood.

Discussion.—From the data submitted it is seen that animals in eight of eleven experiments unquestionably exhibit a spontaneous reticulocytosis after recovery from acute hemorrhage (Table III). This spontaneous reaction always appears after the eleventh week following hemorrhage and in no case has it been seen during the interim. That this is a specific reaction and not a coincidence is proved by the fact that it is completely lacking in the control animals (Fig. 2).

Due to the normal daily fluctuation of the reticulocyte counts in some subjects, it is not always easy to recognize a spontaneous reticulocytosis when it is actually present. (No. 5, Fig. 3). Indeed, without the evidence provided by more pronounced reactions, it is doubtful if such lesser reactions would be recognized at all. However, if, instead of stressing the reticulocyte peak reached in any one day, the volume of red cells being thrown into the circulation over a

TABLE III

SHOWING NUMBER OF EXPERIMENTS AND TYPES OF REACTIONS

Number of experiments reported	13
Number of controls	2
Reaction to hemorrhage	
Number of animals which did not respond typically	3
Number of animals giving spike reaction	3
Number of animals giving sustained reaction	5
Number of spontaneous reactions	8

period of days is computed (No. 1, Fig. 4 and Table IV), such mild reactions as that shown by No. 5, Fig. 3, assumes its proper significance. When this is done it is seen that the *first phase* of the reticulocyte curve always shows a maximum volume of reticulocytes; the *interval period*, a minimum reticulocyte value; and the *terminal* or *spontaneous reaction*, a level somewhere between

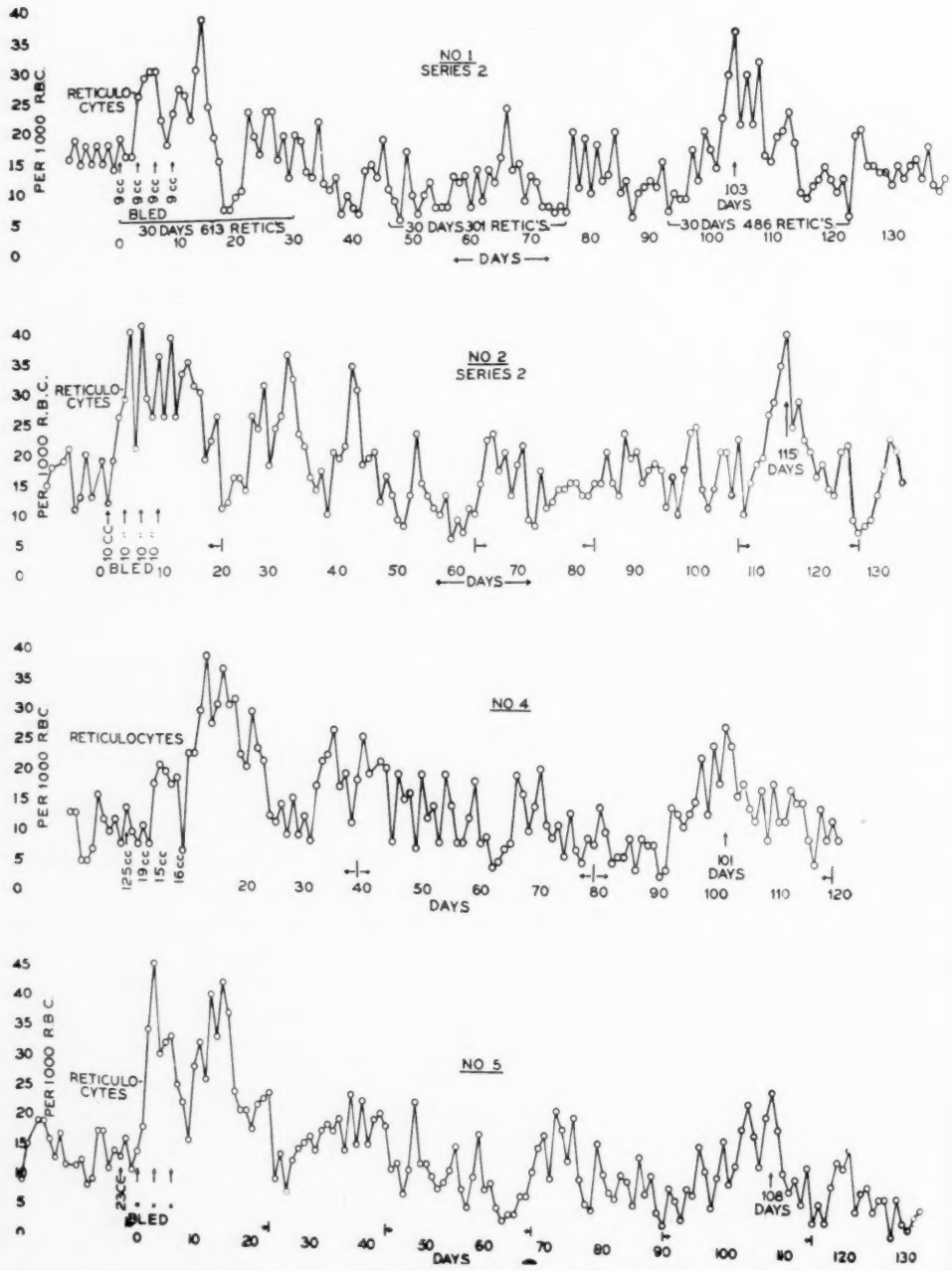

Fig. 4.—Showing reticulocyte counts following four fractional bleedings, each equivalent to approximately .5 per cent of the body weight in blood at intervals of seventy-two hours.

TABLE IV

SHOWING RETICULOCYTE OUTPUT OF EACH ANIMAL (EXCEPT CONTROLS) DURING (1) CONTROL PERIOD, (2) INITIAL REACTION, (3) INTERVAL, AND (4) SPONTANEOUS REACTION*

ANIMAL NO. AND EXPERIMENT NO.	REACTION TYPE	NUMBER OF DAYS UTILIZED IN THE SUBSEQUENT FOUR COMPUTATIONS	RETICULOCYTE OUTPUT DURING PERIOD OF DAYS CITED IN COL. 3							
			OUTPUT AT CONTROL LEVEL OR 100 PER CENT (CELLS)	INITIAL REACTION		INTERVAL OUTPUT		SPONTANEOUS REACTION	
				OUTPUT (CELLS)	PER CENT OF NORMAL	OUTPUT (CELLS)	PER CENT OF NORMAL	OUTPUT (CELLS)	PER CENT OF NORMAL
1	Spike	40	372	681	183	336	98.5	506	136
1, Series 2	Sustained	30	279	613	220	301	108	486	173
2	Control	185	Control	Control	Control	Control	Control	Control	Control
2, Series 2	Sustained	20	178	573	321	287	155	409	229
3	Spike	25	265	490	185	219	71	224	84
4	Sustained	40	400	809	201	509	127	560	140
5	Sustained	25	250	696	278	288	115	362	145
5, Series 2	Sustained	27	270	624	231	261	93	315	117
6	Control	174	Control	Control	Control	Control	Control	Control	Control
7	Spike	40	332	453	136	360	108	343	103
8	Sustained	20	166	376	226	127	76.5	280	169
9	Sustained	42	420	523	125	556	132	461	110
10	Sustained	40	488	548	112	486	100	469	96

*The control output in each case is considered 100 per cent (column 4). The second column in each of the last three divisions shows the percentage deviation from control level. Column 3 shows the number of days covered in each of the four periods evaluated and (except the control periods) are indicated by characters (← →) beneath each curve. The duration of the spontaneous reaction in days was generally the period used in column 3 (seven instances), but in four cases the curves were divided into thirds. The reticulocyte output in columns 4, 5, 7, and 9 represents the sums of the daily values expressed as reticulocytes per 1,000 red cells for the period of days shown in column 3.

those of the first and second periods (Table IV). In some cases the spontaneous reaction, *as recorded in Table IV*, does not seem to have occurred at all, as, for example, in Animal No. 3. Here the percentage of reticulocytes at the time of the spontaneous reaction is only 84 per cent of control level for this particular animal. If, however, the plotted curve of this animal is studied, it is clearly evident that the usual spontaneous reaction has occurred (No. 3, Fig. 3). The ratios of the three phases of the cycle are also maintained.

Not all animals of this series responded in the usual manner to the loss of blood (Table III). Three (Nos. 7, 9, and 10, Fig. 3) gave a different pattern of reaction. All were bled the equivalent of 1 per cent of their body weight in blood at one operation. All gave similar over-all pictures of reticulocyte production in that the output in each case was high in the initial phase (this was typical), remained high during the interval and terminal phases, and tapered off finally (after about 120 days) to approximately control level in cadence with the activity of other animals which reacted typically. Whether the type of hemorrhage used to induce the reaction is responsible for these atypical responses, we are unable to say. Work is in progress to determine the cause.

Life Duration of the Red Blood Cell as Indicated by the Occurrence of Spontaneous Reticulocyte Showers Following Hemorrhage.—Spontaneous reticulocyte showers whose peaks occur within the terminal phases of the reticulocyte curves, 94 to 117 days following hemorrhage, are interpreted as physiologic reactions to the rapid depletions of the red cell populations resulting from

the disappearance (due to age) of the blocks of cells which were suddenly released by the bone marrow in the initial phase of the experiments to replace those removed by hemorrhage.

Some of these replacement cells begin to disappear about eighty days after the induction of the hemorrhage (or after their appearance). The greatest number, however, disappears from ten to twenty-five days later, while a few persist for as long as forty days. This means that there is a spread in the life span of the red cell of from 80 to 120 days, which figures can be derived from the curves in Figs. 3 and 4 by counting the days between the initial and spontaneous reactions.

The values recorded upon the curves of Figs. 3 and 4 at the peak of each spontaneous reaction represent the time lapse between the induction of the hemorrhage and the highest point of the terminal or spontaneous reaction. The figures obtained by this type of experimentation would place the *average life duration* of the red blood cell of the macacus rhesus monkey at about 100 days.

SUMMARY

1. A method is given for making permanent preparations of reticulocytes in monkey blood.

2. Curves are presented showing daily reticulocyte counts in two control and eight experimental animals covering periods of from 120 to 170 days.

3. Two types of blood depletion were employed to stimulate hemopoesis: (1) withdrawal of large amounts (1 per cent of the body weight) at one operation and (2) fractional withdrawal of .5 per cent of the body weight in blood, at intervals of 72 hours (usually four operations).

4. The splan of life of the red blood cell of the macacus rhesus monkey was determined by autonomous blood replacement following hemorrhage. When this method is employed, a spontaneous reticulocyte shower occurs from 94 to 117 days later, indicating a reaction to the mass disappearance of the block of red cells originally mobilized to replace those lost by hemorrhage. The time lapse between the induction of the hemorrhage and the peak of the spontaneous reaction is interpreted to be the life span of the red blood cell in this animal. We realize that from these figures must be deducted the latent period of reaction, which in the monkey was found to be from two to four days.

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OXYURIASIS

Simplified Method of Diagnosis With Glass Slide; Incidence in a Minnesota State Hospital; Result of Treatment With Gentian Violet

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AT THE Willmar (Minnesota) State Hospital it was noted that differential blood counts of patients frequently showed 4 per cent or more eosinophiles. Since routine search for intestinal parasites in such cases usually revealed pinworm infection, we decided to make a complete survey of the entire institution. This was commenced in September, 1940, and completed in July, 1942.

The institution, which is built on the cottage plan, has a capacity of 1,450 beds. Each cottage accommodates about 100 patients. Chronic mental patients, transferred from other hospitals, constitute the great majority of the population. Most of them have been hospitalized for more than a year and many for more than a decade. Although this group is fairly stationary, there are frequent transfers of patients from one cottage to another. Alcoholic patients and drug addicts form the minority. They are admitted directly and, as a rule, remain only from two to four months. Except for those in the hospital at the beginning of the survey, the patients were examined on admission. This group, numbering from forty-five to eighty, was housed largely in a separate ward.

METHOD OF DIAGNOSIS

At first we used the so-called NIH cellophane swab described by Hall.[1] As this was rather cumbersome for survey work, we began to use a strip of cellophane folded over a tongue blade. At the time we did not find mention in the literature of this method. Since this time, however, a somewhat similar technique has been described by Jacobs.[2] The cellophane tape proved as efficient as the NIH swab but still too slow for our purpose. We then commenced to use glass slides with smooth edges of the type ordinarily employed in microscopic work. These were found not only as accurate as the NIH swab and the cellophane tape, but much faster.

A number was etched or scratched on one end of the slide. This served as an identification not only of the slide, but of the exposed side as well. Metal slide holders were made with spacings far enough apart so that the slides could be handled easily without one touching the other. Metal was used for holders in preference to wood because it was easier to clean. The glass slides were cleaned and used repeatedly.

The smears were made sometime between midnight and 4 A.M. No patient to be examined was allowed to take a bath the night before. The patient was asked to bend over and strain slightly. The examiner would hold the marked end of the slide and press the other end against the anal mucosa and mucocutaneous junction in such a manner that one edge of the slide would be toward

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the center of the anus. The slide would then be lifted and the opposite edge pressed against the opposite anal margin. Care was taken to hold the slide in such a manner that only the marked side would be exposed.

It was found advantageous not to have any fecal matter stick to the slide. The moisture of the anal mucosa was sufficient to make the ova adhere to the glass. It was seldom necessary to examine the entire width of the slide. The ova as a rule were found along the edges. If necessary, the slides could be kept for several days before the microscopic examination was made. By the use of low power and a wide eyepiece, a slide could be examined in a few moments, especially when ova were present.

All patients in a cottage were examined the same night. In cases in which no ova were found, the examination was repeated at a later date until the slides from all remaining patients were negative. In some cases as many as eighteen examinations were made. Ova were found fairly frequently in large numbers on slides from patients who repeatedly had been found free from ova.

INCIDENCE

The results for each cottage are given in Table I. Of the 1,871 persons examined, 1,100 (59 per cent) were found to be infected with pinworms. The incidence of the infection was 72 per cent among the chronic mental patients as compared with 1 per cent among the inebriates and none among the employees. Two of the employees, however, had previously been treated for oxyuriasis.

TABLE I

INCIDENCE OF PINWORM INFECTION AND RESULT OF ONE COURSE OF TREATMENT WITH GENTIAN VIOLET

COTTAGE OR GROUP	SEX OF PERSONS EXAMINED	PERSONS EXAMINED	INFECTED BEFORE TREATMENT		INFECTED AFTER ONE COURSE OF TREATMENT	
			NUMBER	PER CENT*	NUMBER	PER CENT†
3	F	83	9	11	0	0
4	M	103	91	88	16	18
5	M	100	40	40	2	5
6	M	106	101	95	6	6
7	F	98	35	36	3	9
8	F	98	69	70	5	7
9	F	84	75	89	7	9
10	F	96	71	74	14	20
11	F	104	94	90	9	10
12	M	102	98	96	1	1
13	M	101	97	96	10	10
14	F	103	89	86	17	19
15	M	99	78	79	3	4
16	M	106	101	95	5	5
Transfers	M	90	38	42	0	0
Transfers	F	53	11	21	0	0
Inebriates	F and M	315	3	1	0	0
Employees	F and M	30	0	0	0	0
Total	F and M	1,871	1,100	59	98	9

*Of persons examined.
†Of persons infected before treatment.

The incidence of oxyuriasis was decidedly higher in the male than in the female population. It was highest in the disturbed and the deteriorated groups. That the condition is not peculiar to the Willmar State Hospital is evidenced by

the incidence among patients received by transfer during the time of the survey. The lower incidence among these is probably due to shorter periods of hospitalization.

TREATMENT

All patients found to be infected were treated with gentian violet. Two ½-grain (0.032 Gm.) enteric-coated tablets were given by mouth twice a day after meals for eight days. After a rest period of eight days, the course was repeated. A third course was given after another eight-day rest period. Thus, 48 grains (3.1 Gm.) of the drug were administered over a period of forty days.

Toxic symptoms due to medication were encountered rarely among the male but fairly frequently among the female patients. These symptoms were anorexia, nausea, vomiting, diarrhea, and mild abdominal pain. Some patients lost weight during the period of treatment. The symptoms disappeared when the medication was suspended for a few days. The therapy was then resumed; either the before-mentioned plan was continued or ½ grain (0.032 Gm.) of the drug was given twice a day continuously for thirty-five days.

After completion of the treatment, search was again made for ova. Six examinations were made in the course of four weeks. Ova were found in ninety-eight (9 per cent of those infected before treatment) after the first course, eleven (1 per cent) after the second, and two (0.2 per cent) after the third. One patient remained infected after five courses of treatment.

The patients in cottages 6 and 12 were re-examined ten months after treatment. Thirty-one (29 per cent) of the former and forty-six (45 per cent) of the latter were found to be infected. Circumstances did not allow us to continue the study. A total of 18,446 slides were examined.

SUMMARY

A simplified method of diagnosing pinworm infection is described. Of 1,871 persons examined in a Minnesota state hospital, 1,100 were infected with pinworms. The incidence of infection was 72 per cent among the chronic mental patients, 1 per cent among the inebriates, and none among the employees. One course of treatment with gentian violet was effective in 91 per cent of cases. Of 208 patients examined ten months after treatment, seventy-seven (37 per cent) were found to be infected.

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A CASE OF TRICHOSTRONGYLUS INFECTION WITH NOTES ON THE IDENTIFICATION OF OVA

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THOUGH various species of Trichostrongylus are frequently found in the intestines of ruminants, instances of human infection occurring in the United States are exceedingly rare. Chandler[1] and Maplestone,[2] on the other hand, reported the incidence varying from 9 to 25 per cent in sheep- and goat-raising localities in India, while Jimbo[3] found 32.3 per cent of 1,215 stools examined at his clinic in Japan positive for Trichostrongylus infection. The species represented in these reports were presumably *Trichostrongylus colubriformis* in the former and definitely *Trichostrongylus orientalis* in the latter. Ransom's[4] predication in 1916, that the parasite may be found in man in continental United States, has not been fully realized. Among a few cases of infection with *Trichostrongylus colubriformis* reported, we may cite Sandground's[5] observation on his own experimental infection and the finding of a single male worm in a surgically removed appendix by Schenken and Moss[6] and identified by Faust.[7] In view of the paucity of human cases of Trichostrongylus infection, it appears to us that the present case is worth reporting. Furthermore, since there is a close resemblance between ova of this parasite and those of hookworms, a description of the characteristic ova seen in this case may help in the differential diagnosis of the two infections.

REPORT OF A CASE

G. P., a laborer, aged 56 years, born in Greece, was admitted to Barnes Hospital with complaints of intermittent pain in the epigastrium and right upper quadrant, chronic constipation, dyspnea, productive cough, headache, ringing of left ear, and a loss of twenty-eight pounds of weight in a year. Physical examination: temperature was 37.5° C.; pulse, 80 per minute; respiration, 20; and blood pressure, 120/76. The patient was dyspneic and the posteroanterior diameter of the chest was increased. There were some inspiratory and expiratory wheezes heard chiefly at the apices of both lungs. The liver was tender, soft, and palpable three fingers below the costal margin. There was a suggestion of a mass extending down from the liver. Bladder dullness was percussed above the symphysis pubis. Laboratory examinations: red blood cell count, 5,120,000 per cubic millimeter; white blood cell count, 6,400; hemoglobin content, 13.2 Gm. per 100 c.c. Differential count was normal with no eosinophiles. Kahn test was positive 4 plus. Blood chemistry: nonprotein nitrogen, 56 mg. per cent; total serum proteins, 6.36 per cent; albumin, 4.26 per cent; globulin, 2.16 per cent. Icterus index was 6. Stool examination revealed the presence of a very few ova of *Trichostrongylus sp.*, detectable only by the use of Willis' levitation technique,[8] and cysts of *Giardia lamblia*. The patient died shortly after admission. The clinical diagnosis of the attending physicians was generalized peritoneal carcinomatosis, substantiated by the examination of sediment of ascitic fluid, senile emphysema of lungs, and latent syphilis. Since autopsy was denied because of religious reason, the species of the parasite remains undetermined. An attempt to culture the organisms from the feces with powdered charcoal in order to secure typical larvae for definite identification was unsuccessful.

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COMMENT ON THE CASE

Clinical observations by several workers, including Jimbo, seem to indicate that the severity of symptoms may be correlated with the number of the parasites localized in the small intestine. The scarcity of adult worms recovered at autopsy as well as the extremely small number of ova usually found in the feces of infected individuals led these investigators to conclude that the great majority of infected individuals are symptom free.

Since the symptoms exhibited by our patient were considered to be attributable to the diseases other than to the parasitic infection, no anthelmintics were administered by the attending physicians. Furthermore, no drugs are known to eradicate the parasite as exemplified in the reports of Jimbo, Sandground, and others. The history of the patient revealed that he had resided in the United States since he migrated from Greece in 1907. He had worked as a farm laborer at various times in the southern states. It is quite conceivable, therefore, that the infection might have been acquired by the accidental ingestion of the third-stage larvae from contaminated soil. According to Chandler, the infection probably occurs more frequently from the ingestion of vegetable matters contaminated with the larvae rather than by penetration through the skin. The possibility that the infection was originally acquired in Greece appears to be remote because of his thirty-six years of residence in this country. In the case of Sandground, the infection was reported to have persisted for as long as eight and one-half years.

THE IDENTIFICATION OF OVA

The diagnosis of Trichostrongylus infection is based on the finding of the characteristic ova in the feces. As previously stated, the detection is usually difficult due to the small number of ova present in the feces, thus necessitating the use of a concentration technique. Moreover, because of a close resemblance of the ova to those of hookworms, the ova of Trichostrongylus were frequently mistaken for the ova of the latter parasite by casual observers. Since ova of various species of Trichostrongylus are indistinguishable one from the other, the definite identification of the species may be accomplished only by the recovery of adult worms following post-mortem examination or by the identity of the typical larvae in the feces-charcoal culture.

TABLE I

MEASUREMENTS OF OVA TRICHOSTRONGYLUS SP.

LENGTH (MICRONS)	FREQUENCY	BREADTH (MICRONS)	FREQUENCY
81	1	40	3
82	1	41	5
83	2	42	4
84	3	44	6
85	3	45	1
86	6	47	1
87	3	49	1
88	2	51	1
90	1		
91	1		
94	1		
97	1		
86 (Average)		43 (Average)	

The ova of *Trichostrongylus sp.* found in the feces of the patient were transparent and elongated oval in shape. As compared with ova of hookworm, they are much longer and slightly wider. The measurements of twenty-five ova taken at random are tabulated in Table I.

As seen from Table I, the ova of *Trichostrongylus sp.* measure 81 to 97 microns in length by 40 to 51 microns in width, averaging 86 by 43 microns. The egg possesses a transparent shell membrane which is thicker and more greenish and lustrous than that of hookworms. While the ends of the ova are more pointed than those of hookworms, in a majority of them one end is somewhat pointed while the other is rounded, as shown in Fig. 1, resembling somewhat the shape of an elongated seedless grape. Maplestone likened it to a hen's egg, while Jimbo compared it to an elongated persimmon when viewed laterally. In some instances the pointed end appears slightly thicker than the

Fig. 1.—Egg of *Trichostrongylus sp.* recovered by the use of Willis levitation technique (×350).

nonpointed end. Ova in freshly voided stool present an advanced stage of segmentation, usually sixteen to twenty-four morula stage of embryonation in staphylococcus-like arrangement. This difference, together with the characteristics described, may be used in differentiating the ova from those of hookworms.

In addition, ova of *Haemonchus contortus*, the parasite of sheep and goats and very rarely of man, possess morphologic characteristics which are indis-

tinguishable from those of Trichostrongylus. Differentiation, therefore, should be made by identifying adult worms or the third-stage larvae.

It is of interest to note in this connection that ova of *Strongyloides stercoralis*, which may rarely appear only in case of profuse diarrhea or following catharsis, may also be mistaken for ova of Trichostrongylus. As a rule, they may be differentiated from the latter by being smaller and by a thinner shell membrane which is less greenish in color and less lustrous. Moreover, in freshly voided stool Strongyloides ova contain embryos ready to be hatched. Ova of *Heterodera radicicola*, which may temporarily appear in feces as result of ingestion of parasitized vegetable materials, require careful scrutiny. These ova may be distinguished from ova of Trichostrongylus by unilateral flattening or slight concavity with rounded ends, though distorted ova of the latter may lead one to an erroneous diagnosis. This difficulty may readily be overcome by repeatedly examining the feces following a vegetable-free diet.

In view of the possibilities of inaccurate diagnosis, the careful examination of the ova of these parasites is of utmost importance due to the fact that many individuals who do not respond to antihookworm therapy may be those passing only ova of Trichostrongylus. That mixed infections of hookworm and Trichostrongylus frequently occur has been verified by the report of Jimbo quoted by Koizumi.[9] Moreover, inaccurate diagnosis may lead one to an erroneous evaluation on the efficacy of drugs for the eradication of hookworm infection.

In areas where hookworm infections are prevalent, therefore, attention should be called to the necessity of carefully conducted examination. The larger size, unique shape, and advanced stage of segmentation at the time of deposition of ova of Trichostrongylus can hardly fail to attract the attention of those who keep them in mind. This will eventually result in uncovering cases of Trichostrongylus infections heretofore either unrecognized or incorrectly diagnosed.

SUMMARY

1. A case of Trichostrongylus infection is presented with the description of the characteristic ova found in the feces.

2. Because of the resemblance of ova of several parasites to those of Trichostrongylus, differential criteria are emphasized to facilitate the differentiation especially from hookworm ova, with which they may be confused by inexperienced observers.

3. The definitive identification of species of strongylate nematodes can be made only by the recovery of the adult worms or the third-stage larvae in culture, since the differentiation on the basis of morphology of ova alone is an impossibility.

4. An immediate need of more careful examination of stools is stressed particularly in localities where endemicity of hookworm infection exists.

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PHOTOSENSITIVITY AS A CAUSE OF FALSELY POSITIVE CEPHALIN-CHOLESTEROL FLOCCULATION TESTS

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SINCE its introduction by Hanger[1] in 1939, the cephalin-cholesterol flocculation test has been widely used as a test of liver function, particularly as a method for detecting the presence of active parenchymatous disease of the liver. However, a disturbing difference in the sensitivity of the reaction has been reported by a number of observers[2-6] and found in our own experience.

This variability in the reaction was recently evidenced by two reports of attempts to design a dilution method for this test. Bruger[4] reported that in his experience the use of sera in various dilutions enabled him to estimate and follow the severity of the hepatic damage. Shortly thereafter Mirsky and Brecht,[5] using the method outlined by Bruger, reported that normal sera were consistently flocculated when used in dilute solution.

Neefe and Reinhold[6] have recently demonstrated that photosensitivity may be a cause of falsely positive cephalin-cholesterol flocculation reactions. They have emphasized the importance of standardizing the technique if the results obtained by various laboratories are to be comparable. In attempting to set up standard conditions, we have confirmed the experience of Neefe on the importance of light in causing falsely positive reactions.

Duplicate tests using the sera of ten normal individuals were made; one tube was kept in total darkness and the other placed outside a laboratory window in direct sunlight. Hanger's method with Difco cephalin-cholesterol mix was used. The results obtained are given in Table I.

The demonstration of the role of sunlight in initiating falsely positive cephalin-cholesterol flocculation tests necessitates that a standardized procedure be adopted if the results obtained by various laboratories are to be comparable. Duplicate samples of five normal sera were tested in an entirely dark cupboard, in direct sunlight, and in a small cupboard containing a 100-watt frosted electric bulb. From the data given in Table II it is evident that both sunlight and artificial light will give falsely positive reactions.

To determine whether the increased temperature induced in sera when they stood in sunlight altered the sensitivity of the test, duplicate specimens were warmed in a water bath at 40° C. for four hours and at 70° C. for thirty minutes (Table III). The results indicate that the falsely positive results are not due to heat. It is interesting to note that in the samples heated to 70° C. for one-half hour only slight flocculation could be obtained in one specimen and none in the other three after standing inside a window for three days. Perhaps alteration of some protein factor at the higher temperature may be responsible for this reaction.

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The patients used in this study were obtained through the courtesy of Dr. A. H. Colwell and the attending staff of the Presbyterian Hospital of the University of Pittsburgh.

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TABLE I

SERUM	DIRECT SUNLIGHT		DARKNESS	
	2 HR.	24 HR.	2 HR.	24 HR.
1	2+	3+	0	0
2	1+	3+	0	±
3	2+	4+	0	0
4	2+	3+	0	0
5	1+	3+	0	0
6	2+	4+	0	0
7	3+	4+	0	0
8	2+	3+	0	0
9	1+	4+	0	±
10	2+	4+	0	0

TABLE II

SERUM	DARKNESS 24 HR.	SUNLIGHT ½ HR.	SUNLIGHT 6 HR.	ARTIFICIAL LIGHT 24 HR.
1	0	0	3+	2+
2	0	0	2+	2+
3	0	±	4+	4+
4	0	0	2+	4+
5	0	0	2+	4+

TABLE III

SERUM	DARKNESS 24 HR.	SUNLIGHT 4 HR.	ARTIFICIAL LIGHT 24 HR.	4 HR. 40° C.	½ HR. 70° C.
1	0	3+	4+	0	0
2	0	2+	4+	0	0
3	0	2+	3+	0	0
4	0	4+	4+	0	0

TABLE IV

SERUM	CONDITION	DARK	LIGHT			
		24 HR.	2 HR.	6 HR.	12 HR.	24 HR.
1	Normal	0	0	1+	2+	3+
2	Normal	0	0	1+	2+	3+
3	Normal	0	0	0	2+	3+
4	Normal	0	0	0	1+	3+
5	Normal	0	0	1+	2+	4+
6	Normal	0	0	1+	2+	3+
7	Normal	0	0	0	2+	3+
8	Normal	0	0	0	1+	3+
9	Normal	0	0	1+	2+	3+
10	Normal	0	0	0	2+	3+
11	Thrombosis cent. vein	0	0	0	2+	3+
12	Disseminating lupus	0	0	0	2+	3+
13	Appendicitis	0	0	0	2+	3+
14	Cerebral arteriosclerosis	0	0	1+	2+	3+
15	Obstructive jaundice	0	0	2+	3+	4+
16	Obstructive jaundice	0	0	0	1+	3+
17	Obstructive jaundice	0	0	1+	2+	3+
18	Hepatitis	3+	2+	4+	4+	4+
19	Hepatitis	4+	2+	4+	4+	4+
20	Hepatitis	3+	1+	2+	3+	4+
21	Hyperthyroid crisis	0	1+	1+	2+	3+
22	Portal cirrhosis	0	2+	3+	4+	4+
23	Portal cirrhosis	0	1+	3+	4+	4+
24	Portal cirrhosis	0	1+	2+	3+	4+
25	Wilson's disease	0	1+	2+	3+	4+
26	Secondary carcinoma of liver	0	1+	2+	3+	4+
27	Multiple myeloma	0	1+	2+	3+	4+

In view of the demonstration of the photosensitivity of the cephalin-cholesterol flocculation test, it has become our practice to run duplicate specimens: one sample read after twenty-four hours in darkness and one read at two, six, twelve, and twenty-four hours in a small (24 by 18 by 16 inches) cupboard containing a 100-watt light bulb. In Table IV are summarized our results to date with this procedure. From these data the impression is gained that the serum from individuals with hepatic disease, although giving a negative cephalin flocculation reaction in the dark, may show abnormal sensitivity to light. Attention is called particularly to Cases 21 to 27 in which hepatic damage was present; the cephalin flocculation reaction in these cases was negative in the dark although showing abnormal sensitivity to light as compared to the controls (Cases 1 to 14).

Obviously if the results obtained by various laboratories are to be comparable, standardization of the light exposure is necessary. In our experience, placing the test tubes in a small cupboard containing a 100-watt light bulb, as noted in the preceding paragraph, has proved satisfactory.

CONCLUSIONS

The photosensitivity of the cephalin-cholesterol flocculation reaction has been confirmed. Since this reaction occurs in artificial light as well as in sunlight, it is not due to any property peculiar to the latter. It is not due to heat generated in the sample by light.

It is suggested that it may be worth while to study the photosensitivity of this reaction in patients with hepatic disease, and a simple method of doing this is given.

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THE KAHN TRIPLE QUANTITATIVE VERIFICATION TECHNIQUE IN THE SEROLOGY OF MAL DEL PINTO (PINTA)

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MENK (1926) found that 74.5 per cent of the sera obtained from sixty-seven patients with mal del pinto, gave a positive reaction with the Wassermann test; 17 per cent were negative and 8.5 per cent were anticomplementary. Gonzalez Herrejon (1927) studied the blood of twenty-three patients with pinta and found twenty-one to be Wassermann positive and two Wassermann negative. Further observations have confirmed the high percentage of positive Wassermann, Kahn, Müller, Hinton, and Chediak tests in patients with mal del pinto. León y Blanco (1940) demonstrated that when the disease becomes generalized, the Wassermann, Kahn, and Müller tests are positive to the extent of 87.56 per cent with a maximum of 97 per cent in the late stages of the disease. Escobar (1940), Gonzalez Guzman (1940), and Briceño Rossi (1943) demonstrated that the Kahn verification test (differential temperature technique; Kahn, 1940) is positive in pinta as it is in syphilis.

In this paper we present the results of the Kahn triple quantitative technique (Kahn, 1942, 1943), which the author considers to have special specific value in the serology of syphilis. Also we present the results of other serologic experiments obtained with sera of patients with mal del pinto.

MATERIAL AND METHODS

Different samples of blood from different localities of the States of Guerrero, Michoacán, and Nayarit were studied. Table I shows the type of the disease in each patient and those patients who were previously treated with arsenic compounds.

The triple quantitative technique for verification reaction was performed using three quantitative procedures: in the first one the serial dilutions were made with distilled water; in the second, the dilutions were made with 0.9 per cent saline solution; and in the third, with 2.5 per cent saline. The Kahn antigen used in all the experiments was the Difco antigen. The standard Kahn test was previously performed in every serum studied.

The globulin and the pseudoglobulin fractions of the blood sera of several patients with pinta were separated by the ammonium sulfate and dialysis method (Wadsworth, 1939). On each one of these fractions the Kahn standard test was performed.

RESULTS

The results are shown in Table I. It can readily be seen that with the more concentrated saline solution used we obtained a stronger positive reaction. Each

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TABLE I

RESULTS OF THE STUDIES OF SERA OF FORTY-SEVEN PATIENTS WITH MAL DEL PINTO

SERUM	TYPE OF PINTO	AGE (YR.)	TREATED	KAHN STD.	TRIPLE QUANTITATIVE VERIFICATION TECHNIQUE			SERUM	TYPE OF PINTO	AGE (YR.)	TREATED	KAHN STD.	TRIPLE QUANTITATIVE VERIFICATION TECHNIQUE		
					DIST. WATER	NaCl 0.9%	NaCl 2.5%						DIST. WATER	NaCl 0.9%	NaCl 2.5%
23	Pintids	12	Yes	++++	+++	++	+++	10	White	32	No	+	++±	+	-
5	Pintids	8	No	-	-	-	-	26	White	15	Yes	++++	±	+	++
24	Pintids	14	Yes	++++	+	±	++	29	White	27	Yes	++	+	++	+++
59	Pintids	8	No	-	-	-	-	32	White	20	Yes	++	±	+	++
1	Blue	16	No	-	+	-	-	42	White	14	Yes	++++	+	++	+++
25	Blue	16	Yes	+++	+	+	++	43	White	35	No	+++	+	++	+++
27	Blue	6	Yes	++++	+	++	+++	48	White	40	Yes	++++	±	+	++
28	Blue	4	Yes	++++	+	++	+++	50	White	40	Yes	+++	+	++	+++
30	Blue	10	Yes	++++	±	+	++	52	White	7	Yes	++	±	-	±
31	Blue	17	Yes	++	+	++	+++	55	White	22	No	+++	±	+	-±
40	Blue	19	Yes	++++	+	++	+++	57	White	20	No	++++	±	+±	++
44	Blue	12	Yes	+++	++	+	++	73	White	30	No	+	-	±	++
45	Blue	45	No	++	+	++	+++	74	White	40	No	++++	±	+	++±
51	Blue	35	Yes	++	±	±	++	75	White	36	No	++	±	++	+++
53	Blue	9	Yes	+++	+	+±	++	2	White and blue	18	Yes	+++	+	++	++++
54	Blue	60	Yes	+	+	++	+++	41	Red	30	Yes	++++	+	++	+++
58	Blue	25	No	++++	±	+	++	46	Blue gray		Yes	++	+	+±	++
78	Blue	34	No	±	±	+	+	49	Blue gray	20	Yes	+	+	++	+++
3	White	30	No	++	±	+	++	47	Brown	22	Yes	++++	+	+±	++
4	White	30	No	++++	++	++±	+++	21	Brown		Yes	+	±	++	+++
6	White	12	No	+++	+++	+	+++±	79	Brown	4	Yes	±	+	±	±
7	White	10	Yes	-	-	-	-	80	Brown	7	Yes	+++	+	+±	++
8	White	12	No	+++	+	-±	+++	81	Brown	8	Yes	±	±	+	+±
9	White	13	No	+++	±	++	+++								

- = Negative.

column in Table I represents the average flocculation intensity read in four Kahn tubes containing serum dilutions of 1/5, 1/10, 1/20, and 1/40, respectively.

The standard Kahn tests with the euglobulin and pseudoglobulin fractions separated from sera of patients with pinta were positive for both fractions.

SUMMARY

The different concentrations of sodium chloride from 0 to 2.5 per cent, applied in Kahn's triple quantitative verification technique, increase the intensity of the reaction in the sera of patients with mal del pinto.

The globulin and pseudoglobulin fractions of sera of these patients reacted in a similar way, both giving positive standard Kahn tests.

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NITROGEN BALANCE ON A RESTRICTED CALORIC INTAKE

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DURING early studies on intravenous protein feeding with amino acids[1] we always tried to provide all of the energy requirements by giving sufficient glucose. To supply 1,600 calories parenterally means an intravenous injection lasting seven hours inasmuch as a normal adult can utilize only 60 Gm. per hour. Moreover, this would mean 4,000 c.c. of 10 per cent glucose or 8,000 c.c. of 5 per cent glucose. Because of the obvious practical difficulties, we began to question the necessity of supplying all of the energy requirements, during short periods of time at least. Quite to our surprise we found that positive nitrogen balance could be maintained in many of those patients even though the amount of glucose administered fell far short of that required for full energy needs. Obviously the rest of the calories were being obtained from tissue fat. In order to study this matter further, a series of experiments was carried out in dogs to determine how well nitrogen balance can be maintained on a restricted caloric but adequate protein intake.

PREVIOUS OBSERVATIONS

Lusk[2] states that under conditions of complete starvation 87 per cent of the caloric requirements are met by tissue fat, the rest coming from tissue protein, which seems to offer ample theoretical justification for relying on tissue fat for much of the caloric needs. Indeed, Rubner[3] was able to cut the caloric requirements by 33 per cent without disturbing the degree of nitrogen balance. On the other hand, Thomas[4] found that nitrogen balance could not be maintained on a pure protein diet; yet it is obvious that the Eskimos maintain health and growth on a diet which is largely protein with some fat but little or no carbohydrate. Block[5] studied nitrogen balance in three obese and three normal women for consecutive seven-day periods during which the caloric intake was reduced from 80 to 20 per cent of the basal requirement, although the protein intake was maintained at 1 Gm. or more per kilogram per day. Positive nitrogen balance was observed in twenty of twenty-four periods. Moreover, the weight loss in the obese patients was exactly as predicted on the basis that the adipose tissue furnished the calories not ingested in the diet. In a previous report from this laboratory[6] dogs were shown to retain much more nitrogen on isocaloric but inadequate diets in which 80 per cent of the calories were protein as compared with those in which 80 per cent of the calories were carbohydrate.

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PRESENT OBSERVATIONS

Four healthy female mongrel dogs were selected for these experiments. They were prepared for easy catheterization by dividing the posterior vaginal wall. After healing had occurred they were observed two at a time. Previous to the beginning of each series of observations, the dogs had been on a full well-balanced diet and were well nourished.

The dietary intake during the experiment was rigidly controlled by gavage feedings. No other food was permitted except that water was allowed ad libitum. They were kept in metabolism cages for the collection of urine and were catheterized at the beginning of each period which in general lasted four days. The diet consisted of dextrimaltose and Amigen, which is an enzymic hydrolysate of casein and pork pancreas. Two different mixtures were prepared consisting of (a) 80 per cent carbohydrate and 20 per cent protein (as Amigen) and (b) 80 per cent protein (as Amigen) and 20 per cent carbohydrate. The two dietary constituents were mixed in a small quantity of water and given by gavage once a day. This procedure worked very well and provoked vomiting only twice in one animal and is the only occasion in which consecutive observations were not carried out (Table II). Otherwise, the experiments were without event, including the absence of diarrhea. The stools were collected in the early experiments but were found to be quite scanty and to comprise less than 5 per cent of the total nitrogen in the urine and were therefore discontinued.

The daily amount of food administered was set at either 50 calories per kilogram or 25 calories per kilogram, based on 4 calories per gram of carbohydrate as dextrimaltose and 3 calories per gram of Amigen. In calculating the caloric value of Amigen, its nitrogen content was used as the base; this was multiplied by 6.25, giving its presumed protein equivalent. Because Amigen contains only 12 per cent instead of the 16 per cent nitrogen in whole protein, a figure of 3 calories per gram of Amigen instead of the 4 calories for whole protein was obtained. Inasmuch as a figure of 3.7 calories per gram of Amigen was obtained by direct bomb calorimetry,[7] this figure may be incorrect. If the figure of 3.7 were taken instead of 3.0, the difference would involve the number of calories given in each group of experiments. Thus the caloric intake instead of being 50 calories in each animal would be 52 in the low protein and 58 in the high protein mixture. At a 25 calorie intake, the difference would be 26 as against 29. This slight discrepancy, however, would probably not influence the main findings even if this calculation were justified. Nitrogen was determined by a micro-Kjeldahl technique described by Sobel, Yuska, and Cohen.[8]

EXPERIMENTAL FINDINGS

The observations made are recorded in Tables I to III. It will be noted that when the dietary intake consisted of 80 per cent carbohydrate and 20 per cent protein (as Amigen), nitrogen balance was achieved at a level of 50 calories per kilogram in only one of the two dogs. Moreover, when the caloric intake was cut in half in these dogs, a negative balance was found in each of the two animals (Table I). By contrast, positive nitrogen balance was obtained even at a 25 calories per kilogram intake in similar experiments in two other dogs in which the proportion was reversed so as to contain 80 per cent protein (as

TABLE I

NITROGEN (N) BALANCE ON AN INTAKE OF 80 PER CENT CARBOHYDRATE AND 20 PER CENT PROTEIN (AS AMIGEN)

CALORIC INTAKE DURING PERIODS I TO III, 50/KG.; DURING PERIODS IV TO VII, 25/KG.

PERIOD (4 DAYS)	DOG L (WEIGHT, 12.4 KG.)				DOG S (WEIGHT, 7.2 KG.)			
	INTAKE		OUTPUT IN URINE	NITROGEN BALANCE	INTAKE		OUTPUT IN URINE	NITROGEN BALANCE
	CH (GM.)	N_1 (GM.)	N_2 (GM.)	N_1-N_2 (GM.)	CH (GM.)	N_1 (GM.)	N_2 (GM.)	N_1-N_2 (GM.)
I	500	20	24.88	-4.88	288	11.4	11.32	+0.08
II	500	20	20.92	-0.92	288	11.4	10.72	+0.68
III	500	20	20.16	-0.16	288	11.4	9.44	+1.96
Average per day				-0.49				+0.23
IV	250	10	14.04	-4.04	144	5.7	8.00	-2.30
V	250	10	11.64	-1.64	144	5.7	6.88	-1.18
VI	250	10	11.76	-1.76	144	5.7	6.96	-1.26
VII	250	10	11.72	-1.72	144	5.7	7.40	-1.70
Average per day				-0.57				-0.40

TABLE II

NITROGEN (N) BALANCE ON AN INTAKE OF 20 PER CENT CARBOHYDRATE AND 80 PER CENT PROTEIN (AS AMIGEN)

CALORIC INTAKE DURING PERIODS I TO III, 50/KG.; DURING PERIODS IV TO VI, 25/KG.

PERIOD (4 DAYS)	DOG B (WEIGHT, 9.3 KG.)				DOG T (WEIGHT, 10.5 KG.)			
	INTAKE		OUTPUT IN URINE	NITROGEN BALANCE	INTAKE		OUTPUT IN URINE	NITROGEN BALANCE
	CH (GM.)	N_1 (GM.)	N_2 (GM.)	N_1-N_2 (GM.)	CH (GM.)	N_1 (GM.)	N_2 (GM.)	N_1-N_2 (GM.)
I	93	59.52	51.12	+8.40	*105	67.2	72.04	-4.84
II	93	59.52	54.28	+5.24	*105	67.2	59.24	+7.96
III	93	59.52	56.72	+2.80	---	---	----	----
Average per day				+1.37				+0.39
IV	47	29.76	30.68	-0.92	53	33.6	34.52	-0.92
V	47	29.76	28.60	+1.16	53	33.6	32.40	+1.2
VI	47	29.76	27.40	+2.36	53	33.6	31.76	+1.84
Average per day				+0.22				+0.19

*Periods not consecutive due to vomiting.

TABLE III

NITROGEN (N) BALANCE ON AN INTAKE OF 25 CALORIES PER KILOGRAM

PERIODS I TO IV ON DOG T AND PERIOD V ON DOG B, INTAKE WAS 80 PER CENT CARBOHYDRATE AND 20 PER CENT PROTEIN (AS AMIGEN); PERIODS I TO IV ON DOG B AND PERIOD V ON DOG T, INTAKE WAS 20 PER CENT CARBOHYDRATE AND 80 PER CENT PROTEIN (AS AMIGEN)

PERIOD (4 DAYS)	DOG T (WEIGHT, 9.7 KG.)				DOG B (WEIGHT, 9.4 KG.)			
	INTAKE		OUTPUT IN URINE	NITROGEN BALANCE	INTAKE		OUTPUT IN URINE	NITROGEN BALANCE
	CH (GM.)	N_1 (GM.)	N_2 (GM.)	N_1-N_2 (GM.)	CH (GM.)	N_1 (GM.)	N_2 (GM.)	N_1-N_2 (GM.)
I	194	7.6	14.60	-7.0	47	30.0	29.32	+0.68
II	194	7.6	11.16	-3.56	47	30.0	24.68	+5.32
III	194	7.6	9.12	-1.52	47	30.0	24.60	+5.40
IV	194	7.6	9.12	-1.52	47	30.0	26.48	+3.52
Average per day				-0.85				+0.93
V (5 days)	60	38.0	29.4	+8.6	235	9.4	15.75	-6.35
Average per day				+1.7				-1.27

Amigen) and 20 per cent carbohydrate (Table II). This same effect was observed more strikingly in the next two experiments in which the animals were placed immediately on a 25 calorie per kilogram intake. In one the proportion was 80 per cent carbohydrate and 20 per cent protein, which produced a persistent average negative balance of 0.85 Gm. per day for four consecutive four-day periods, whereas in an immediately following five-day period, in which the proportion was reversed, pronounced positive balance of 1.7 Gm. per day was achieved. In the other experiment in which the 25 calories consisted of 80 per cent protein (as Amigen) and 20 per cent carbohydrate, positive nitrogen balance of nearly a gram a day was consistently maintained in four consecutive four-day periods, whereas in an immediately following five-day period in which the proportion was reversed, a negative balance of 1.27 Gm. per day was observed (Table III).

COMMENT

From the findings herein presented it would seem that good nitrogen balance can be achieved by simply increasing greatly the proportion of nitrogen in the diet, even though the caloric intake is far from adequate. This has certain practical implications. For example, the necessity of supplying all of the energy requirements during short periods of intravenous feeding assumes less significance, and the surgeon is safely relieved of the practical difficulty of supplying as much glucose as was hitherto deemed necessary. It is probable that a relatively small amount of glucose will permit adequate utilization of protein for short periods of time at least. Undoubtedly the rest of the caloric needs are met by use of tissue fat.

These findings have a further implication in wartime in that members of the Armed Forces are not infrequently separated from their sources of supply and must for certain periods maintain themselves on a restricted ration, which leads to loss of body tissue. Because part of the caloric needs may be met by tissue fat which contributes little to physiologic performance, it is much more important to maintain protein tissue which is of great physiologic significance. From this it may be inferred that priority should be given to protein rather than to caloric intake. Because the present observations show that positive nitrogen balance can be maintained on a low caloric intake when sufficient protein is provided, it would seem obvious that such a ration would result in greater physiologic efficiency than one consisting largely of carbohydrate and is associated with a negative nitrogen balance. In other words, with a restricted diet consisting purely of carbohydrate and protein, a better metabolic and physiologic result will follow when the proportion allotted to carbohydrate is reduced and that allotted to protein is increased, in contrast to the normal distribution in a well-balanced diet.

SUMMARY

In dogs on restricted isocaloric intake, positive nitrogen balance can be maintained when the proportion of protein to carbohydrate is 4 to 1 but not 1 to 4, respectively. The practical implications of these findings in regard to the desirability in restricted diets of giving priority to protein over caloric needs are discussed.

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LIMITATIONS OF THE CORRELATION BETWEEN THE RED CELL VOLUME AND HEMOGLOBIN OF BLOOD DURING PREGNANCY

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IT HAS been rather generally accepted that the red cell volume of the blood and the hemoglobin are proportional and that one value may be checked by the other. A number of exceptions discovered among pregnant patients led us to undertake an investigation of the true value of this possible correlation during the course of pregnancy.

In a series of consecutive determinations made on seventy-five pregnant women upon admission to the prenatal dispensary, using oxalated blood, we found Pearson's correlation to be 0.677 between the red cell volume and the hemoglobin. In a second series of thirty-seven patients, using heparinized blood, we found a correlation of 0.747. This means that while there is a definite correlation, of proved significance, in either series, one is not justified in predicting one value from a determination of the other, even when using heparinized blood. This is illustrated in Fig. 1, which shows the individual distribution of the hemoglobin-cell volume relations. The two lines indicate true proportionalties, based on the hemoglobin and cell volume averages, and coincide with those drawn to indicate the tendency toward proportion, in either series, as shown by the distribution of the individual findings. It is clear, from Fig. 1, that while a certain correlation does exist, individual determinations show too great a variance, in both directions, for it to be applicable in individual cases.

The cell volumes were found by spinning the oxalated or heparinized blood at high speed until there was no further packing of the cells; then the volume of the latter is given in per cent of the volume of the whole blood. For the oxalated series we found a mean value of 33.4 per cent, with a standard error of 0.41. The computed range, from these figures, to cover individual determinations, is from 22.7 to 44.1 per cent. With the heparinized blood the mean value was 38.5 per cent, with a standard error of 0.64, giving a computed range of 26.9 to 50.2 per cent.

Hemoglobin was determined by the Fisher electrophotometer, using the method described by Evelyn,* 50 cu. mm. of blood in 10 c.c. of water, with 1 drop of strong ammonium hydroxide added immediately before reading, using a 525 millimicron filter. For the combined series of 112 determinations, we found a mean of 11.43 Gm. hemoglobin per 100 c.c. of blood, with a standard error of 0.11. The computed range for individual determinations would be from 7.8 Gm. to 15.0 Gm. per 100 c.c.

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*Evelyn, K. A.; Determination of Oxyhemoglobin, J. Biol. Chem. **115**: 63, 1936.

SUMMARY

Correlations of 0.677 and 0.747 were found between the red cell volume and hemoglobin of blood from pregnant women, using potassium oxalate and heparin, respectively, as anticoagulants. Variations were found in about 25 per cent of the cases, in both directions, as shown in Fig. 1.

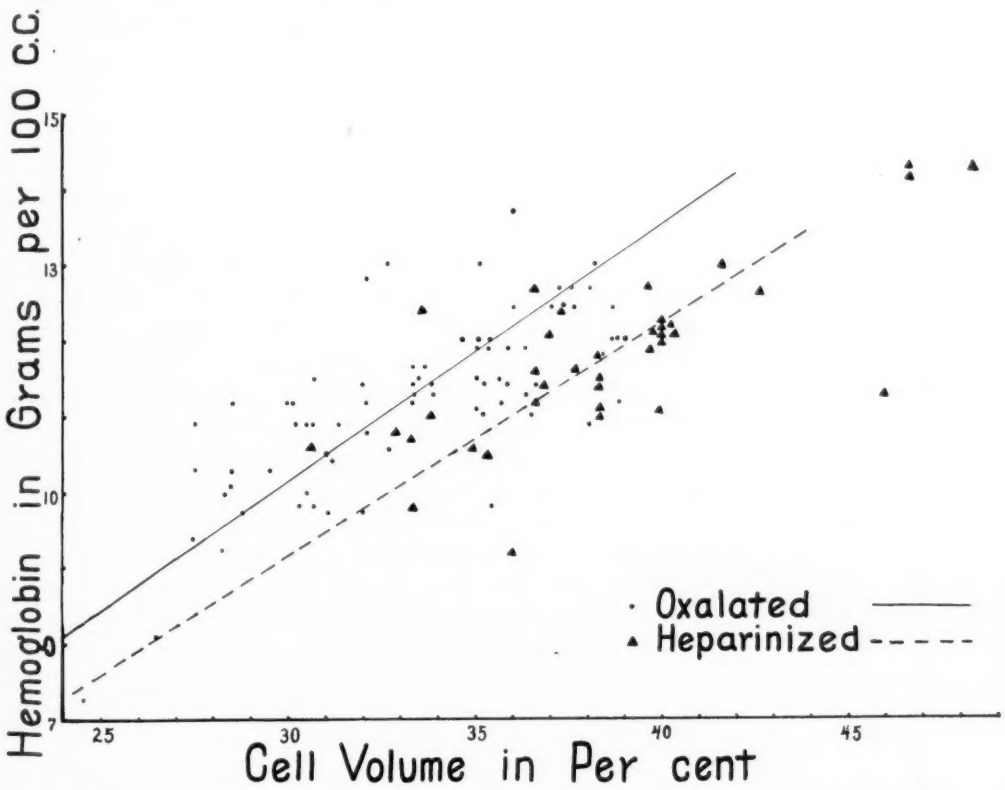

Fig. 1.—Distribution of the individual hemoglobin-cell volume determinations on pregnant women.

Mean values, with computed ranges to cover individual findings, were established for the red cell volumes and hemoglobin concentration in blood from pregnant women.

LABORATORY METHODS

A METHOD FOR THE COLORIMETRIC DETERMINATION OF POTASSIUM IN BIOLOGIC PRODUCTS

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IN UNDERTAKING a study on the effect of protein and amino acid deficiency states on the mineral metabolism of the human being and experimental animals, it became necessary to find a satisfactory, yet rapid, procedure for the estimation of potassium in the urine and tissue digests. Our experiments with a number of the many procedures available for this analysis demonstrated that on modification the colorimetric technique reported by Blanchetière and Pirlot[1] was extremely well suited to our purposes. This method is based on the measurement of potassium as the green color produced by the basic carbonate of cobalt which is obtained by transforming the insoluble potassium sodium cobaltinitrite into the respective chlorides of the complex cations and treatment of the mixture with bicarbonate. Our modification of the procedure involved an improvement in the over-all accuracy by establishment of optimal precipitation conditions for the cobaltinitrite complex and increased speed of operation achieved by the elimination of what proved to be a long and unnecessary dehydration period for the conversion of the cobalt complex into the chlorides by the use of concentrated hydrochloric acid. The adequacy of these modifications was demonstrated by the quantitative recovery of potassium added to urine specimens and tissue digests.

In order to obtain base-line data for our subsequent studies on human subjects fourteen twenty-four hour urine specimens from seven normal adult males maintained on unrestricted normal diets were analyzed for potassium and nitrogen content. These were found to contain from 2.92 to 4.76 Gm. of potassium with the nitrogen potassium ratios varying from 2.51 to 4.05 for the series.

Application of the method to human serum and red blood cells yield values which compare favorably with those reported in the literature. Analysis of neutralized acid digests of eight whole immature rats (70 to 125 Gm.) showed the potassium content to be 0.61 ± 0.04 per cent.

EXPERIMENTAL

Reagents.—

Sodium cobaltinitrite reagent:[2] To a solution of 25 Gm. of cobalt nitrate dissolved in 62.5 c.c. of 20 per cent acetic acid (by volume) in a 1 liter Erlenmeyer flask are cautiously added 210 c.c. of a solution of sodium nitrite prepared by dissolving 120 Gm. of the salt in 180 c.c. of water. After the vigorous evolution of nitrogen oxide gases which ensues has subsided, the remainder

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of the gases are removed by drawing air through the solution. The reagent is then filtered and stored in the icebox where it keeps well for three or four months.

Ethyl alcohol: 30 per cent solution by volume.

Hydrochloric acid: 90 per cent solution by volume.

Potassium bicarbonate: Saturated solution.

Hydrogen peroxide: 3 per cent solution prepared by 1:10 dilution of Superoxol.

Potassium standard: 94.8 mg. of oven-dried (100°) potassium chloride are dissolved in 100 c.c. of distilled water; 1 c.c. of this solution is equivalent to 0.5 mg. of potassium.

PROCEDURE

Preservation of Urine.—Twenty-four hour specimens were collected in brown bottles containing 50 c.c. of 15 per cent HCl (by volume) and 1 c.c. of 10 per cent alcoholic thymol and were made to a uniform volume of 2 liters before removal of the sample for potassium determination. It has been found that under these conditions the potassium content of the specimens remains unchanged after storage for one week or more at room temperature.

Method.—To 1 or 2 c.c. of urine, or samples (pH 5-6) containing not more than 3 mg. of potassium, in a 15 c.c. graduated conical centrifuge tube are added 2 c.c. of the cobaltinitrite reagent and distilled water to make a final reacting volume of 5 c.c. After the mixtures have been allowed to stand for at least two hours at room temperatures, the tubes are centrifuged for ten minutes at 3,000 r.p.m. and the supernatant solutions discarded by careful decantation. The yellow precipitates are washed twice by successive resuspension in 5 c.c. of 30 per cent ethanol, centrifugation, and decantation of the supernatant fluid. The tubes are inverted on to filter paper and drained for ten or fifteen minutes; then 0.5 c.c. of 90 per cent hydrochloric acid is added to each sample and the precipitates are dissolved and nitrogen oxide gases driven off by heating gently over a Fischer microburner. The blue test solutions are cooled in tap water, and on the addition of 0.5 c.c. of 3 per cent hydrogen peroxide a deep pink color appears. Now saturated potassium bicarbonate solution is added dropwise until effervescence ceases and the solutions turn to a deep emerald green color. The samples are all made to the 10 c.c. mark with bicarbonate solution and read in the Klett-Summerson photoelectric colorimeter using the S-60 filter. As a check on the satisfactory performance of these operations, 1 c.c. and 5 c.c. aliquots of the potassium standard are run simultaneously with each set of urine samples. Since the color intensity shows no tendency to fade in two or three hours, it is possible to perform as many as twelve duplicate determinations in each run without danger of error from this source.

Calculations.—The amount of potassium in the samples can be readily calculated from the following formula:

$$\frac{\text{Reading of sample}}{\text{Reading of standard}} \times \text{mg. of K in standard} = \text{mg. of K in sample}$$

Or, since the color reaction obeys Beer's law, the potassium content of a given sample can be estimated directly from a previously prepared calibration curve.

RESULTS

In order to assess the quantitative effect of the modifications made in the reaction conditions of the method, suitable aliquots of the potassium standard were submitted to the manipulations previously described. The linear relationship of the color intensity to the amount of potassium which is obtained by the use of the method (Fig. 1) is interpreted as evidence of the suitability of the modifications and validity of Beer's law for the color reaction.

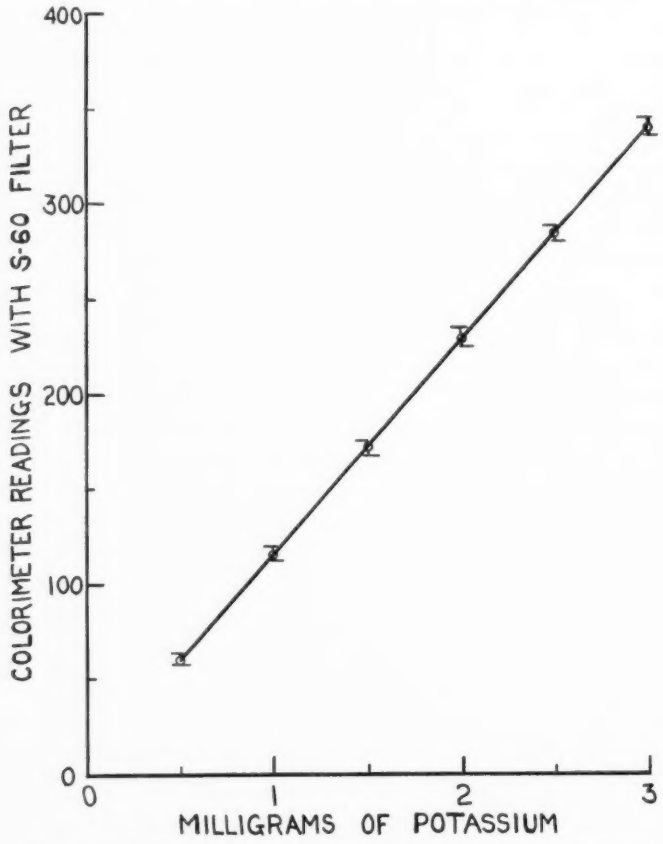

Fig. 1.—Relation of color intensity to amount of potassium. Each point represents the average of ten determinations. The bars above and below each point indicate the deviation range of the readings.

The applicability of the procedure to the urine was indicated by the quantitative recovery of added potassium (Table I).

The results of the nitrogen[3] and potassium analysis of twenty-four hour specimens of seven normal adults on unrestricted normal diets are shown in Table II. When due consideration is given to the qualitative and quantitative variations of the food intake which prevailed, the N/K ratio would not seem to fluctuate too greatly either for the individual or the group.

The analyses of 1 and 2 c.c. samples of blood serum from six different normal children subsequent to ashing in a muffle furnace for twenty-four hours at 500° C. gave values ranging from 14.4 to 21.8 mg. per 100 c.c. The potassium

content of adult human erythrocytes was determined by ashing 200 to 500 mg. samples of carefully washed and dried cells in the muffle furnace and found to be 0.44 per cent. These figures are well within the range of those reported by other investigators.[4]

TABLE I

RECOVERY OF ADDED POTASSIUM

SAMPLE	POTASSIUM ADDED (MG.)	POTASSIUM FOUND (MG.)	RECOVERY OF ADDED POTASSIUM (PER CENT)
Urine A, 1 c.c.	0	1.33	
Urine A, 1 c.c.	0.53	1.90	102.0
Urine B, 1 c.c.	0	1.78	
Urine B, 1 c.c.	0.53	2.30	99.5

TABLE II

DAILY OUTPUT OF POTASSIUM AND NITROGEN OF NORMAL MALES ON NORMAL DIET

SUBJECT	BODY WEIGHT (KG.)	FIRST WEEK			SECOND WEEK		
		TOTAL K (GM.)	TOTAL N (GM.)	N/K RATIO	TOTAL K (GM.)	TOTAL N (GM.)	N/K RATIO
4	79.5	3.30	12.95	3.92	4.26	11.48	2.69
5	63.6	2.92	10.75	3.68	3.22	10.60	3.29
6	65.9	3.16	11.40	3.61	3.23	10.90	3.38
7	77.4	3.90	10.48	2.68	4.24	10.60	2.51
8	71.9	3.57	12.40	3.48	3.56	12.90	3.62
9	100.0	4.76	17.30	3.63	3.82	14.91	3.91
10	79.5	4.76	17.20	3.62	3.34	13.54	4.05

COMMENT

The accuracy of potassium determinations based on gravimetric or titrimetric or colorimetric elaborations of the cobaltinitrite precipitation has been the subject of much discussion. The errors inherent to the gravimetric methods arise from variations in potassium content and solubility of the complex. Hubbard[5] and Jendrassik[6] have described schemes to overcome the variability in composition. Solubility errors prompted Breh and Gaebler[6] to precipitate potassium as silver potassium cobaltinitrite which is presumably less soluble than the sodium analog. In spite of these modifications, unanimity of opinion on the validity of gravimetric data does not seem to have been reached as yet. Inasmuch as in our method the color readings are made with reference to a potassium standard which has been submitted to operations identical to those of the unknown, it is not necessary to consider the effect of composition variations of the complex or to correct for its solubility. That these factors remain constant in our procedure can be adduced from the reproducibility of the calibration curve and recovery test data.

The titrimetric estimation of potassium by the use of acid permanganate has been criticized principally on the score that the precipitates may contain organic substances which would result in high values. Devices to circumvent this difficulty have been described by Hubbard[5] and Leulier,[8] but they are laborious and of doubtful efficacy.

Of the many inorganic and organic color reactions reported in the literature for the assay of potassium in terms of the cobalt or nitrite content of the complex, that of Blanchetière and Pirlot[1] was selected by us because it is the least

affected by variations in reagents quantities and yields the most stable color. The use of artificial standards is obviously not to be recommended as these would introduce the composition and solubility errors discussed below.

Calculations from Clark's[9] data show that the adult male retains from 200 to 180 mg. of potassium per day of an average intake of 2.47 Gm. of potassium. The experiments of Loeb and associates[10] confirm these figures and further reveal that the retention is not greatly affected by an increase in the potassium input. In view of the relatively small potassium requirements of the human adult, it is obvious from our excretion data that the average American diet supplies an abundance of potassium. It is of further interest to note that our potassium excretion values are in good agreement with the calculated average potassium content of the American daily diets.[4]

SUMMARY

A simple and accurate colorimetric method for the estimation of potassium in biologic materials has been described. Application of the method to urine of seven normal adult males on normal diets showed that the daily potassium output varies from 2.92 to 4.76 Gm. The analyses of human blood serum and red blood cells gave values which are in accord with those found in the literature.

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THE OCCURRENCE OF HIGH BLOOD BLANKS IN THE COLORIMETRIC DETERMINATION OF SULFONAMIDES

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Holbourn and Pattle[1] have recently claimed that in their modification of the dimethyl-α-naphthylamine method[2] for estimating sulfanilamide it is not necessary to remove the excess nitrite. Under our conditions it has been found, however, that this method may give very high blank values for normal blood (rat, rabbit, and beef), especially when hydrolyzed with HCl. When high blood dilutions, such as 1:200, are used, the blank values for normal filtrates hydrolyzed with HCl have reached 50 mg. per cent, expressed as sulfanilamide.

A purple color is produced, the intensity of which increases indefinitely. This is apparently due to impurities in the coupling agent, for Marshall[3] states: "A high blank value may be obtained with certain samples of dimethyl-α-naphthylamine due to contamination with α-naphthylamine." We have found that destruction of the excess nitrite with urea, or with ammonium sulfamate as in Marshall and Litchfield's[2] method, results in relatively steady readings and low blanks.

Method.—Normal blood was hemolyzed in distilled water and precipitated with trichloracetic acid at a final concentration of 3 per cent. Hydrolysis was carried out by heating the filtrates for one hour in the water bath in the presence of 1 c.c. of 4 *N* HCl or H_2SO_4 per 20 c.c. of filtrate. Readings were taken in a photoelectric colorimeter with a green filter ten and fifteen minutes after the addition of the coupling agent. The various methods were standardized against sulfanilamide, using the ten-minute readings.

The coupling agents, dimethyl-α-naphthylamine and N-(1-naphthyl)-ethylenediamine dihydrochloride, were obtained from the Eastman Kodak Co. All other chemicals were of reagent grade. In the modified Holbourn and Pattle method, urea was added as 0.5 c.c. of 40 per cent solution, and ammonium sulfamate as 0.5 c.c. of 0.5 per cent solution, three minutes after the addition of nitrite. After a further two-minute period the coupling agent was added.

Results.—Typical of our results with various blood samples are the values given in Table I for blanks on normal rabbit blood diluted 1:25. The values are expressed as equivalent sulfanilamide concentrations in the filtrates, reagent blanks having been deducted from the blood blanks.

When dimethyl-α-naphthylamine is used and the excess nitrite is destroyed, the blank values are low and the readings are virtually constant. The values compare favorably with those obtained in the Bratton and Marshall method[4] using N-(1-naphthyl)-ethylenediamine. Lee, Hannay, and Hand[5] have claimed that in this latter method the sulfamate may be omitted if alcohol is added. They recommend the use of H_2SO_4 for hydrolysis, and Table I shows that by this method the blank values are extremely low; when HCl is used, however, the

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blanks are high. We have observed that steady readings are not obtained unless at least 0.2 mg. per cent of sulfanilamide is present. However, the errors are small when ten minutes is taken as the standard time, and the absence of gas bubbles makes this method very convenient.

TABLE I

BLANK VALUES ON NORMAL RABBIT BLOOD DILUTED 1:25
(EXPRESSED AS μG. SULFANILAMIDE PER 100 C.C. FILTRATE)

METHOD	REAGENT		BLOOD FILTRATE		HYDROLYZED FILTRATE	
	10 MIN.	15 MIN.	10 MIN.	15 MIN.	10 MIN.	15 MIN.
Holbourn and Pattle[1]	54	65	47	64	73	93
Holbourn and Pattle[1] plus urea	41	38	2	3	5	5
Holbourn and Pattle[1] plus sulfamate	38	36	2	1	4	5
Bratton and Marshall[4]	5	5	3	3	4	4
Lee, Hannay, and Hand[5]	26	24	-2	-3	0	-2
Lee, Hannay, and Hand[5] hydrolyzed with HCl	--	--	--	--	24	23

SUMMARY

In the colorimetric determination of sulfonamides with dimethyl-α-naphthylamine and with N-(1-naphthyl)-ethylenediamine, normal blood filtrates may give very high blank values unless the excess nitrite is destroyed with urea or sulfamate. The blanks are especially high when blood filtrates are hydrolyzed with HCl.

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NEW TYPE OF NEEDLE HOLDER*

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IN DOING venepunctures at the Red Cross Blood Donor Center a small quantity of novocain is injected with a sterile hypodermic needle before the venepuncture needle is inserted. Where 900 to 1,000 donors are bled daily a large number of hypodermic needles are required since a fresh sterile needle is used for each donor. To have these needles readily available for use has constituted quite a problem. Many of the Blood Donor Centers use individual test tubes

Fig. 1.—*A*, Needle holder 25 by 85 mm.; *B*, spring lock cover; *C*, crystal dish 90 by 50 mm.; *D*, crystal dish cover 100 by 50 mm.

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*This needle holder was made for the Chicago Blood Donor Center by The Helmco Corporation, 1215 West Fullerton Ave., Chicago 14, Ill.

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for each needle, but that involves a great deal of work both in preparing the needles for autoclaving and in removing the needles from the test tubes.

At the Chicago Blood Donor Center cork needle holders were used, each needle holder containing 40 needles and being covered by a crystal dish 100 by 50 mm. This was a time-saver but had the disadvantage that after repeated autoclaving the cork would deteriorate and cause plugging of many of the needles. Also, the cost of discarded cork holders and the increasing difficulty in securing the cork were additional factors in prompting us to search for a method to overcome these objections. As a result a new type of needle holder has been developed.

The new needle holder (Fig. 1), which is now in use at the Blood Donor Center of the Chicago Chapter of the American Red Cross, was constructed of spun copper, chromium plated, with a special brass supporting knob with a snap-on lock feature accommodating the spring lock cover. This type of needle holder offers many advantages in that it is light, durable, and easily sterilized. It holds 50 needles size 25-G, $\frac{5}{8}$ of an inch in length.

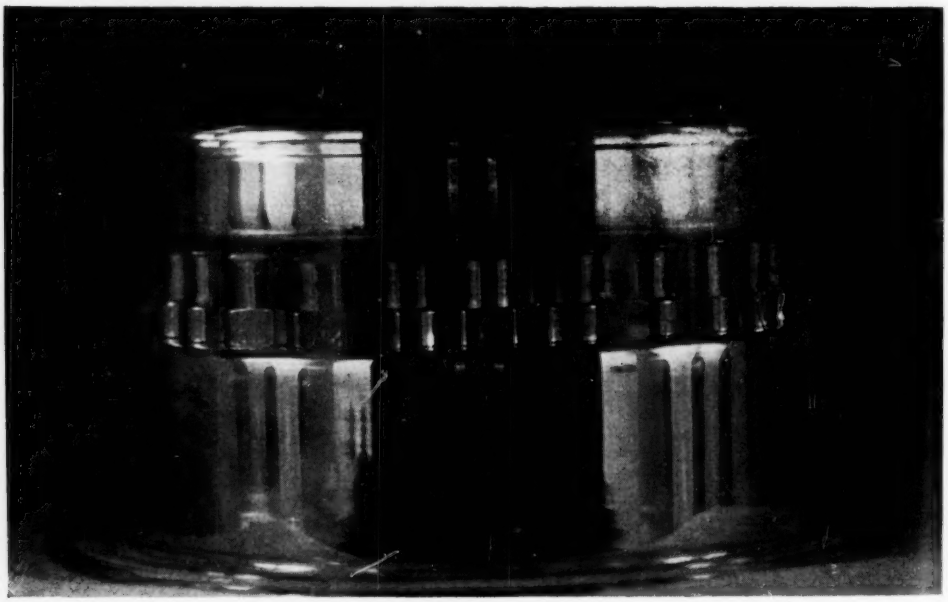

Fig. 2.—Needle holder assembled.

The chromium needle holder (Fig. 1, *A*) is placed in a crystal dish (Fig. 1, *C*) 90 by 50 mm. The needles are placed in individual perforations with points down and covered with a chromium-plated lid with a spring lock (Fig. 1, *B*) which holds the needles in position, and the entire holder is covered with a crystal dish 100 by 50 mm. (Fig. 1, *D*) and then autoclaved. For use on the mobile units a piece of gauze is interposed between the bottom and top crystal dish so as to avoid breakage of these dishes in transit.

The needle holder is placed on the working stand between two bleeding tables; the weight of the needle holder is sufficient to keep it from sliding around on the table. The crystal dish cover is lifted, the chromium lid removed, and

the crystal cover replaced. In use the cover is raised with one hand and a sterile syringe containing novocain is inserted into the upright shank of the needle, which is removed without touching any of the adjacent needles, and the crystal dish cover is then replaced. A similar procedure is carried out in each individual case.

This needle holder has been in use at the Blood Donor Center in Chicago for the past four months where approximately 100,000 venepunctures have been done with a large varying personnel. It has been found to be a simple and time-saving method for changing needles. It has been satisfactory in overcoming the objection of the cork needle holders. It is our opinion that physicians performing mass immunizations or allergists performing numerous intra-dermal tests might find this type of needle holder useful. Its depth could be varied to hold needles of other lengths.

BOOK REVIEWS

Outline of the Amino Acids and Proteins. Edited by *Melville Sahyun*, M.A., Ph.D., Vice President and Director of Research, Frederick Stearns and Company, Detroit, Mich., and thirteen contributing authors. Reinhold Publishing Corporation, New York, N. Y. Price $4.00. Cloth with 260 pages, illustrated.

It is now generally appreciated that amino acids and proteins play both a unique and essential role in life processes. In planning the make-up of the book, the author takes the commendable position that if one is to understand the manifold functions of amino acids and proteins, a knowledge of their occurrence, composition, physical chemical properties, and behavior is essential. The subject matter is presented in twelve short and concise chapters. The first six chapters deal with the discovery of the amino acids; the occurrence, amino acid content, and properties of proteins; the structure of the protein molecule and the hydrolysis of proteins; the synthesis and isolation of certain amino acids; and the methods of analysis for amino acids and proteins. The last six chapters consider the relation of amino acids and their derivatives to immunity; the relation of amino acids to biologically important products and the role of certain amino acids in detoxication; the metabolism of proteins and amino acids and the intermediary metabolism of individual amino acids; the significance of amino acids and proteins in nutrition; and nitrogen equilibrium and the biological value of proteins.

It is appreciated that in recent years several comprehensive texts dealing with the chemistry of the amino acids and proteins have appeared. The present book does not aim to cover all phases of the subject nor does it attempt to treat any subject exhaustively. It is written with the purpose of outlining in a simple and readable manner the essentials of the chemistry and biochemistry of the amino acids and proteins and to serve as a background for those who wish to go into the subject more deeply. In this respect the book would appear to have a definite place and should be well received by students and teachers of biochemistry as well as by clinicians desiring readable information on the subject.

E. M.

Manual of Clinical Mycology. Prepared under the auspices of the Division of Medical Sciences of the National Research Council. By *Norman F. Conant*, Ph.D., Assistant Professor of Bacteriology, Duke University School of Medicine, and Mycologist to Duke Hospital; *Donald Stover Martin*, M.D., Associate Professor of Bacteriology and Associate in Medicine, Duke University School of Medicine; *David Tillerson Smith*, M.D., Professor of Bacteriology and Associate Professor of Medicine, Duke University School of

Medicine; *Roger Denio Baker,* M.D., Associate Professor of Pathology, in charge of Surgical Pathology, Duke University School of Medicine; and *Jasper Lamar Calloway,* M.D., Assistant Professor of Medicine, in charge of Dermatology and Syphilology, Duke University School of Medicine. W. B. Saunders Co., Philadelphia and London, 1944. Price $3.50. Cloth, 5¼ by 7¾ inches with 348 pages and 148 figures.

This book follows the style of other military medical manuals published by the Saunders Company particularly for the Armed Forces. It is small, compact, well illustrated, well printed, and has few typographical errors. The 148 figures make up 11 world maps which show the distribution of some of the mycoses; 19 roentgenographs; 37 illustrations; and 197 photographs of mycologic, clinical, and pathologic aspects of the diseases.

Each of the five authors was responsible for certain sections which were incorporated in each chapter and the whole rewritten and coordinated by Donald S. Martin. Norman F. Conant contributed the mycology section; Donald S. Martin, the section on geographic distribution and immunology; David T. Smith prepared the symptomatology, differential diagnosis, prognosis, and treatment of the systemic diseases; Roger D. Baker was responsible for the pathology; and Jasper L. Calloway contributed the clinical aspects of the dermatomycoses and other superficial mycoses. There are twenty-five chapters, the last two dealing with the fundamentals of elementary mycology and contaminants. The appendix contains a formulary and deals with methods of study. The index is complete.

The recognition of the importance of mycotic infections is borne out by the amazing increase in publications of case reports, experimental investigations, and, more recently, textbooks. The controversies as to the nomenclature of fungi, however, still exist. In this respect, the authors have tried to simplify the identification and classification of pathogenic fungi. In so doing, however, many of the commonly accepted names have been eliminated or reduced to synonymy with other lesser known names with the result that more confusion will exist. Examples of this are *Trichophyton purpureum,* which is referred to as *T. rubrum,* and *Achorion Schönleinii,* which is changed to *Trichophyton Schönleinii. Paracoccidiodes brasiliensis, P. cerebriformis,* and *P. tenuis,* the agents of South American blastomycosis, are all transferred to the incorrect, but still maintained genus Blastomyces as the single species *B. brasiliensis.* The genus Actinomyces is used only for the anaerobic, gram-positive and non-acid-fast organisms represented by the single species, *A. bovis,* whereas the aerobic forms which are also Gram-positive and some of which are acid-fast are called Nocardia. Such a separation can be very confusing, especially in view of the work of some investigators who find that oxygen requirements can be reversible with this group of organisms.

To simplify the identification of fungi in the laboratory, the authors recommend the use of blood agar incubated at 37° C. Unfortunately, such a procedure, if at all useful, can be so only in the hands of a trained mycologist and should not be relied upon for species identification.

The clinical discussions for most of the systemic mycoses are well presented, although brief for some diseases. In contrast, the methods of treatment for

some of the mycoses are numerous and might be confusing to the clinician. The use of vaccine desensitization is stressed, especially in certain patients with blastomycosis. The known immunologic phenomena are well presented although the value of skin testing with fungous extracts in many mycoses is considered either as doubtful or nonspecific. The sections on pathology emphasize particularly the appearance of the organism in tissue but also present most of the salient features of the pathologic processes. Unfortunately, not all of the diseases described have sections on pathology.

This book should be a valuable addition to one's library. It is easy to handle, inexpensive, and contains recent information both for the clinician and for the laboratory technician.

Etiology, Diagnosis and Treatment of Amebiasis. By *Charles F. Craig,* M.D., M.A. (Hon.), F.A.C.S., F.A.C.P., Colonel, U.S.A., Retired, D.S.M.; Late Commandant, Army Medical School, and Assistant Commandant, Army Medical Center, Washington, D.C.; Emeritus Professor of Tropical Medicine, Medical School, Tulane University of Louisiana, New Orleans, La. Williams & Wilkins Company, Baltimore, 1944. Price $4.50. Cloth with 332 pages.

This is a priceless encyclopedia on the important subject of Amebiasis written by the world's renowned authority. Every phase of the subject is systematically presented with the usual clearness and conciseness which characterize Colonel Craig's writings. It contains all recent and valuable information accumulated in numerous researches since the first publication of the author's treatise on *Amebiasis and Amebic Dysentery* in 1934. These additions, together with those preceding, which have undergone the author's careful and painstaking scrutiny, based on his wide personal experience, are the highlights of the book.

We may say that Amebiasis would seem sufficient for the title of the book without specialization, as every phase of the subject is included.

The book is strongly recommended to all those engaged in clinical and laboratory medicine.

H. T.

Aids to Clinical Pathology Including Postmortem Technique. By *David Haler,* M.B., B.S. (Hons.) London, D.C.P. London, Hon. Pathologist, All Saints' Hospital, London, and Pathologist, Emergency Medical Service; Member of L.C.C. Panel of Special Pathologists; Williams & Wilkins Co., Baltimore, 1944. Cloth with 358 pages.

The Reticulo-Endothelial System in Sulfonamide Activity. By *Frank Thomas Maher,* Ph.D., Assistant Professor of Pharmacognosy and Pharmacology. University of Illinois Press, Urbana, Ill. Price $2.50. Paper bound with 232 pages and 23 figures.